BEST AVAILABLE IMAGES

Defective images within this document are accurate representation of The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Search Results -

Terms	Documents
l9 and ((424/93.21)!.CCLS.)	5

Database: US Patents Full-Text Database
US Pre-Grant Publication Full-Text Database
JPO Abstracts Database
EPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Refine Search: `l9 and ((424/93.21 )!.CCLS. )` Clear

Search History

Today's Date: 11/21/2001

DB Name	Query	Hit Count	Set Name
USPT,PGPB	l9 and ((424/93.21)!.CCLS.)	5	L17
USPT,PGPB	l9 and ((424/93.2)!.CCLS.)	4	L16
USPT,PGPB	l9 and ((435/377)!.CCLS.)	3	L15
USPT,PGPB	l9 and ((435/373)!.CCLS.)	5	L14
USPT,PGPB	l9 and ((435/372)!.CCLS.)	7	L13
USPT,PGPB	l9 and ((435/368)!.CCLS.)	8	L12
USPT,PGPB	l9 and ((435/455)!.CCLS.)	4	L11
USPT,PGPB	l9 and ((435/325)!.CCLS.)	14	L10
USPT,PGPB	l6 and l8	61	L9
USPT,PGPB	l7 and culture and lineage	845	L8
USPT,PGPB	l2 and l5 and differentia$	6780	L7
USPT,PGPB	l1 and l5	130	L6
USPT,PGPB	l3 or l4	47823	L5
USPT,PGPB	sox or sox1 or sox2 or sox3	932	L4
USPT,PGPB	selectable near5 marker or antibiotic$	46929	L3
USPT,PGPB	embryonic adj (stem or germ or carcinoma) or es or eg or ec	1279990	L2
USPT,PGPB	multipotential near5 cell	234	L1

WEST

Generate Collection

Search Results - Record(s) 1 through 5 of 5 returned.

1. Document ID: US 20010033834 A1

L17: Entry 1 of 5 File: PGPB Oct 25, 2001

PGPUB-DOCUMENT-NUMBER: 20010033834
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010033834 A1

TITLE: Pleuripotent stem cells generated from adipose tissue-derived stromal cells and uses thereof

PUBLICATION-DATE: October 25, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wilkison, William O.	Bahama	NC	US	
Gimble, Jeffrey	Chapel Hill	NC	US	

US-CL-CURRENT: 424/93.7; 424/93.21, 435/325, 435/366, 435/368, 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

2. Document ID: US 20010007659 A1

L17: Entry 2 of 5 File: PGPB Jul 12, 2001

PGPUB-DOCUMENT-NUMBER: 20010007659
PGPUB-FILING-TYPE: new-utility
DOCUMENT-IDENTIFIER: US 20010007659 A1

TITLE: USE OF LENTIVIRAL VECTORS FOR ANTIGEN PRESENTATION IN DENDRITIC CELLS

PUBLICATION-DATE: July 12, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
WONG-STAAL, FLOSSIE	SAN DIEGO	CA	US	
LI, XINGIANG	SAN DIEGO	CA	US	
KAN-MITCHELL, JUNE	RANCHO SANTA FE	CA	US	

US-CL-CURRENT: 424/93.21; 424/93.2, 435/320.1, 514/44

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 5888814 A

L17: Entry 3 of 5 File: USPT Mar 30, 1999

US-PAT-NO: 5888814
DOCUMENT-IDENTIFIER: US 5888[redacted] A

TITLE: Recombinant host cells encoding TNF proteins

DATE-ISSUED: March 30, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kriegler; Michael	San Francisco	CA		
Perez; Carl F.	Berkeley	CA		

US-CL-CURRENT: 435/360; 424/93.1, 424/93.21, 424/93.7, 435/252.3, 435/320.1, 435/325, 435/363, 435/366, 435/371, 435/372 , 435/372.1, 435/69.5, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

4. Document ID: US 5874077 A

L17: Entry 4 of 5 File: USPT Feb 23, 1999

US-PAT-NO: 5874077
DOCUMENT-IDENTIFIER: US 5874077 A

TITLE: Human til cells expressing recombinant TNF prohormone

DATE-ISSUED: February 23, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kriegler; Michael	San Francisco	CA		
Perez; Carl F.	Berkeley	CA		

US-CL-CURRENT: 424/93.21; 435/372.3, 435/69.7

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

5. Document ID: US 5785964 A

L17: Entry 5 of 5 File: USPT Jul 28, 1998

US-PAT-NO: 5785964
DOCUMENT-IDENTIFIER: US 5785964 A

TITLE: Three-dimensional genetically engineered cell and tissue culture system

DATE-ISSUED: July 28, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 424/93.21; 424/93.1, 424/93.2, 424/93.3, 435/320.1, 435/325, 800/14

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

Terms	Documents
19 and ((424/93.21)!.CCLS.)	5

Display 20 Documents, starting with Document: 5

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 4 of 4 returned.

1. Document ID: US 20010007659 A1

L16: Entry 1 of 4 File: PGPB Jul 12, 2001

PGPUB-DOCUMENT-NUMBER: 20010007659
PGPUB-FILING-TYPE: new-utility
DOCUMENT-IDENTIFIER: US 20010007659 A1

TITLE: USE OF LENTIVIRAL VECTORS FOR ANTIGEN PRESENTATION IN DENDRITIC CELLS

PUBLICATION-DATE: July 12, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
WONG-STAAL, FLOSSIE	SAN DIEGO	CA	US	
LI, XINGIANG	SAN DIEGO	CA	US	
KAN-MITCHELL, JUNE	RANCHO SANTA FE	CA	US	

US-CL-CURRENT: 424/93.21; 424/93.2, 435/320.1, 514/44

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

2. Document ID: US 5861290 A

L16: Entry 2 of 4 File: USPT Jan 19, 1999

US-PAT-NO: 5861290
DOCUMENT-IDENTIFIER: US 5861290 A

TITLE: Methods and polynucleotide constructs for treating host cells for infection or hyperproliferative disorders

DATE-ISSUED: January 19, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goldsmith; Mark A.	West Roxbury	MA	02132	
Ralston; Robert O.	San Francisco	CA	94122	

US-CL-CURRENT: 424/93.2, 435/320.1, 514/44, 536/23.1, 536/23.2, 536/23.5, 536/23.53, 536/23.6, 536/23.7, 536/23.72, 536/24.1, 536/24.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 5837510 A

L16: Entry 3 of 4 File: USPT Nov 17, 1998

US-PAT-NO: 5837510
DOCUMENT-IDENTIFIER: US 5837[redacted] A

TITLE: Methods and polynucleotide constructs for treating host cells for infection or hyperproliferative disorders

DATE-ISSUED: November 17, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goldsmith; Mark A.	San Francisco	CA	94131	
Ralston; Robert O.	San Francisco	CA	94122	

US-CL-CURRENT: 435/455; 424/93.2, 435/320.1, 435/456, 514/44, 536/23.1, 536/23.2, 536/23.5, 536/23.53, 536/23.6, 536/23.7, 536/23.72, 536/24.1, 536/24.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

4. Document ID: US 5785964 A

L16: Entry 4 of 4 File: USPT Jul 28, 1998

US-PAT-NO: 5785964
DOCUMENT-IDENTIFIER: US 5785964 A

TITLE: Three-dimensional genetically engineered cell and tissue culture system

DATE-ISSUED: July 28, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 424/93.21; 424/93.1, 424/93.2, 424/93.3, 435/320.1, 435/325, 800/14

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

Generate Collection

Terms	Documents
l9 and ((424/93.2)!.CCLS.)	4

Display 20 Documents, starting with Document: 4

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 3 of 3 returned.

☑ 1. Document ID: US 6001654 A

L15: Entry 1 of 3 File: USPT Dec 14, 1999

US-PAT-NO: 6001654
DOCUMENT-IDENTIFIER: US 6001654 A

TITLE: Methods for differentiating neural stem cells to neurons or smooth muscle cells using TGT-.beta. super family growth factors

DATE-ISSUED: December 14, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Shah; Nirao M.	New York	NY		

US-CL-CURRENT: 435/377; 435/325, 435/352, 435/353, 435/368, 435/375

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 2. Document ID: US 5919702 A

L15: Entry 2 of 3 File: USPT Jul 6, 1999

US-PAT-NO: 5919702
DOCUMENT-IDENTIFIER: US 5919702 A

TITLE: Production of cartilage tissue using cells isolated from Wharton's jelly

DATE-ISSUED: July 6, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Purchio; Anthony F.	La Jolla	CA		
Naughton; Brian A.	El Cajon	CA		
San Roman; Julia	San Diego	CA		

US-CL-CURRENT: 435/378; 424/93.1, 435/325, 435/366, 435/377

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 3. Document ID: US 5175103 A

L15: Entry 3 of 3 File: USPT Dec 29, 1992

US PATENT NO: 5175103

DOCUMENT-IDENTIFIER: US 51751[redacted] A

TITLE: Preparation of pure cultures of post-mitotic human neurons

DATE-ISSUED: December 29, 1992

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lee; Virginia	Philadelphia	PA		
Pleasure; Samuel	Philadelphia	PA		

US-CL-CURRENT: 435/455; 435/377

Full | Title | Citation | Front | Review | Classification | Date | Reference

KWIC | Draw Desc | Image

Generate Collection

Terms	Documents
19 and ((435/377)!.CCLS.)	3

Display 20 Documents, starting with Document: 3

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 5 of 5 returned.

1. Document ID: US 6225048 B1

L14: Entry 1 of 5 File: USPT May 1, 2001

US-PAT-NO: 6225048
DOCUMENT-IDENTIFIER: US 6225048 B1

TITLE: Dendritic-marker expressing macrophage cultures and methods of reactivating latent virus

DATE-ISSUED: May 1, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Soderberg-Naucler; Cecilia E.	Hagarsten			SEX
Fish; Kenneth N.	San Diego	CA		
Moses; Ashlee	Portland	OR		
Streblow; Daniel	Tigard	OR		
Nelson; Jay	Tualatin	OR		

US-CL-CURRENT: 435/5; 435/235.1, 435/325, 435/373

Full | Title | Citation | Front | Review | Classification | Date | Reference KWIC | Draw Desc | Image

2. Document ID: US 5858721 A

L14: Entry 2 of 5 File: USPT Jan 12, 1999

US-PAT-NO: 5858721
DOCUMENT-IDENTIFIER: US 5858721 A

TITLE: Three-dimensional cell and tissue culture system

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/69.1; 435/1.1, 435/320.1, 435/325, 435/326, 435/347, 435/363, 435/365.1, 435/366, 435/367, 435/368, 435/369, 435/370, 435/371, 435/372, 435/373, 435/396, 435/397, 435/398, 435/399, 435/402, 435/69.2, 435/69.3, 435/69.4, 435/69.5, 435/69.6, 435/70.1

Full | Title | Citation | Front | Review | Classification | Date | Reference KWIC | Draw Desc | Image

3. Document ID: US 5580781 A

L14: Entry 3 of 5 File: USPT Dec 3, 1996

US-PAT-NO: 5580781
DOCUMENT-IDENTIFIER: US 5580781 A

TITLE: Three-dimensional tumor cell and tissue culture system

DATE-ISSUED: December 3, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/1.1; 424/443, 424/529, 424/530, 424/534, 424/572, 424/93.7, 435/29, 435/32, 435/34, 435/347, 435/373 , 435/395, 435/399, 435/402

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

4. Document ID: US 5518915 A

L14: Entry 4 of 5 File: USPT May 21, 1996

US-PAT-NO: 5518915
DOCUMENT-IDENTIFIER: US 5518915 A

TITLE: Three-Dimensional mucosal cell and tissue culture system

DATE-ISSUED: May 21, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 424/422; 424/484, 424/572, 435/1.1, 435/284.1, 435/29, 435/32, 435/371, 435/373, 435/399

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

5. Document ID: US 5516681 A

L14: Entry 5 of 5 File: USPT May 14, 1996

US-PAT-NO: 5516681
DOCUMENT-IDENTIFIER: US 5516681 A

TITLE: Three-dimensional pancreatic cell and tissue culture system

DATE-ISSUED: May 14, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/353; 424/422, 424/484, 424/572, 435/1.1, 435/1.2, 435/284.1, 435/29, 435/32, 435/347, 435/373

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

Generate Collection

Terms	Documents
l9 and ((435/373)!.CCLS.)	5

Display 20 Documents, starting with Document: 5

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 7 of 7 returned.

1. Document ID: US 20010033834 A1

L13: Entry 1 of 7 File: PGPB Oct 25, 2001

PGPUB-DOCUMENT-NUMBER: 20010033834
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010033834 A1

TITLE: Pleuripotent stem cells generated from adipose tissue-derived stromal cells and uses thereof

PUBLICATION-DATE: October 25, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wilkison, William O.	Bahama	NC	US	
Gimble, Jeffrey	Chapel Hill	NC	US	

US-CL-CURRENT: 424/93.7; 424/93.21, 435/325, 435/366, 435/368, 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

2. Document ID: US 6242579 B1

L13: Entry 2 of 7 File: USPT Jun 5, 2001

US-PAT-NO: 6242579
DOCUMENT-IDENTIFIER: US 6242579 B1

TITLE: Antigen found on a small subset of human hematopoietic cells which binds to monoclonal antibody MG1

DATE-ISSUED: June 5, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lawman; Michael J. P.	Key Largo	FL	33037	
Lawman; Patricia	Key Largo	FL	33037	

US-CL-CURRENT: 530/395; 435/343.2, 435/372, 530/388.75, 530/827

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 6074639 A

L13: Entry 3 of 7 File: USPT Jun 13, 2000

US-PAT-NO: 6074639
DOCUMENT-IDENTIFIER: US 6074 A

TITLE: Ex vivo expansion of hematopoietic cells using interleukin-3 (IL-3) variant fusion proteins

DATE-ISSUED: June 13, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bauer; S. Christopher	New Haven	MO		
Abrams; Mark Allen	St. Louis	MO		
Braford-Goldberg; Sarah Ruth	St. Louis	MO		
Caparon; Maire Helena	Chesterfield	MO		
Easton; Alan Michael	Maryland Heights	MO		
Klein; Barbara Kure	St. Louis	MO		
McKearn; John Patrick	Glencoe	MO		
Olins; Peter O.	Glencoe	MO		
Paik; Kumnan	Ballwin	MO		
Thomas; John Warren	Town & Country	MO		

US-CL-CURRENT: 424/93.71; 424/85.1, 424/85.2, 435/372, 435/372.2, 435/372.3, 435/69.52

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

☑ 4. Document ID: US 5997860 A

L13: Entry 4 of 7 File: USPT Dec 7, 1999

US-PAT-NO: 5997860
DOCUMENT-IDENTIFIER: US 5997860 A

TITLE: Ex-vivo expansion of stem cells using combinations of interleukin-3 (IL-3) variants and other cytokines

DATE-ISSUED: December 7, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bauer; S. Christopher	New Haven	MO		
Abrams; Mark Allen	St. Louis	MO		
Braford-Goldberg; Sarah Ruth	St. Louis	MO		
Caparon; Maire Helena	Chesterfield	MO		
Easton; Alan Michael	Maryland Heights	MO		
Klein; Barbara Kure	St. Louis	MO		
McKearn; John Patrick	Glencoe	MO		
Olins; Peter O.	Glencoe	MO		
Paik; Kumnan	Ballwin	MO		
Thomas; John Warren	Town & Country	MO		

US-CL-CURRENT: 424/93.71; 424/85.1, 424/85.2, 424/93.7, 435/372, 435/372.2, 435/372.3

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

☐ 5. Document ID: US 5888814 A

L13: Entry 5 of 7 File: USPT Mar 30, 1999

US-PAT-NO: 5888814
DOCUMENT-IDENTIFIER: US 5888[redacted] A

TITLE: Recombinant host cells encoding TNF proteins

DATE-ISSUED: March 30, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kriegler; Michael	San Francisco	CA		
Perez; Carl F.	Berkeley	CA		

US-CL-CURRENT: 435/360; 424/93.1, 424/93.21, 424/93.7, 435/252.3, 435/320.1, 435/325, 435/363, 435/366, 435/371, 435/372 , 435/372.1, 435/69.5, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

6. Document ID: US 5858721 A

L13: Entry 6 of 7 File: USPT Jan 12, 1999

US-PAT-NO: 5858721
DOCUMENT-IDENTIFIER: US 5858721 A

TITLE: Three-dimensional cell and tissue culture system

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/69.1; 435/1.1, 435/320.1, 435/325, 435/326, 435/347, 435/363, 435/365.1, 435/366, 435/367, 435/368, 435/369, 435/370, 435/371, 435/372, 435/373, 435/396, 435/397, 435/398, 435/399, 435/402, 435/69.2, 435/69.3, 435/69.4, 435/69.5, 435/69.6, 435/70.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

7. Document ID: US 5266480 A

L13: Entry 7 of 7 File: USPT Nov 30, 1993

US-PAT-NO: 5266480
DOCUMENT-IDENTIFIER: US 5266480 A

TITLE: Three-dimensional skin culture system

DATE-ISSUED: November 30, 1993

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/371; 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

Generate Collection http://westbrs:8820/bin/gate.exe?f=TOC&s...2q43o.14&ref=13&dbname=USPT,PGPB&ESNAME=

Terms	Documents
19 and ((435/372)!.CCLS.)	7

Display 20 Documents, starting with Document: 7

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 8 of 8 returned.

1. Document ID: US 20010038836 A1

L12: Entry 1 of 8 File: PGPB Nov 8, 2001

PGPUB-DOCUMENT-NUMBER: 20010038836
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010038836 A1

TITLE: Application of myeloid-origin cells to the nervous system

PUBLICATION-DATE: November 8, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
During, Matthew	Philadelphia	PA	US	
Leone, Paola	Philadelphia	PA	US	

US-CL-CURRENT: 424/93.7; 435/368

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

2. Document ID: US 20010033834 A1

L12: Entry 2 of 8 File: PGPB Oct 25, 2001

PGPUB-DOCUMENT-NUMBER: 20010033834
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010033834 A1

TITLE: Pleuripotent stem cells generated from adipose tissue-derived stromal cells and uses thereof

PUBLICATION-DATE: October 25, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wilkison, William O.	Bahama	NC	US	
Gimble, Jeffrey	Chapel Hill	NC	US	

US-CL-CURRENT: 424/93.7; 424/93.21, 435/325, 435/366, 435/368, 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 6001654 A

L12: Entry 3 of 8 File: USPT Dec 14, 1999

US-PAT-NO: 6001654
DOCUMENT-IDENTIFIER: US 6001 A

TITLE: Methods for differentiating neural stem cells to neurons or smooth muscle cells using TGT-.beta. super family growth factors

DATE-ISSUED: December 14, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Shah; Nirao M.	New York	NY		

US-CL-CURRENT: 435/377; 435/325, 435/352, 435/353, 435/368, 435/375

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

☐ 4. Document ID: US 5858721 A

L12: Entry 4 of 8 File: USPT Jan 12, 1999

US-PAT-NO: 5858721
DOCUMENT-IDENTIFIER: US 5858721 A

TITLE: Three-dimensional cell and tissue culture system

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/69.1; 435/1.1, 435/320.1, 435/325, 435/326, 435/347, 435/363, 435/365.1, 435/366, 435/367, 435/368, 435/369, 435/370, 435/371, 435/372, 435/373, 435/396, 435/397, 435/398, 435/399, 435/402, 435/69.2, 435/69.3, 435/69.4, 435/69.5, 435/69.6, 435/70.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

☑ 5. Document ID: US 5849553 A

L12: Entry 5 of 8 File: USPT Dec 15, 1998

US-PAT-NO: 5849553
DOCUMENT-IDENTIFIER: US 5849553 A

TITLE: Mammalian multipotent neural stem cells

DATE-ISSUED: December 15, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/467; 435/320.1, 435/325, 435/353, 435/368, 435/455, 435/462, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

6. Document ID: US 5672499 A

L12: Entry 6 of 8 File: USPT Sep 30, 1997

US-PAT-NO: 5672499
DOCUMENT-IDENTIFIER: US 5672499 A

TITLE: Immoralized neural crest stem cells and methods of making

DATE-ISSUED: September 30, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/353; 435/320.1, 435/325, 435/368, 435/467, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

7. Document ID: US 5654189 A

L12: Entry 7 of 8 File: USPT Aug 5, 1997

US-PAT-NO: 5654189
DOCUMENT-IDENTIFIER: US 5654189 A

TITLE: Preparation of pure cultures of post-mitotic human neurons

DATE-ISSUED: August 5, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lee; Virginia	Philadelphia	PA		
Pleasure; Samuel	Philadelphia	PA		

US-CL-CURRENT: 435/368

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

8. Document ID: US 5654183 A

L12: Entry 8 of 8 File: USPT Aug 5, 1997

US-PAT-NO: 5654183
DOCUMENT-IDENTIFIER: US 5654183 A

TITLE: Genetically engineered mammalian neural crest stem cells

DATE-ISSUED: August 5, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/456; 435/320.1, 435/325, 435/353, 435/368, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

Generate Collection

Terms	Documents
l9 and ((435/368)!.CCLS.)	8

Display 20 Documents, starting with Document: 8

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 4 of 4 returned.

1. Document ID: US 5928947 A

L11: Entry 1 of 4 File: USPT Jul 27, 1999

US-PAT-NO: 5928947
DOCUMENT-IDENTIFIER: US 5928947 A

TITLE: Mammalian multipotent neural stem cells

DATE-ISSUED: July 27, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/455; 424/93.7, 435/325, 435/440, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

2. Document ID: US 5849553 A

L11: Entry 2 of 4 File: USPT Dec 15, 1998

US-PAT-NO: 5849553
DOCUMENT-IDENTIFIER: US 5849553 A

TITLE: Mammalian multipotent neural stem cells

DATE-ISSUED: December 15, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/467; 435/320.1, 435/325, 435/353, 435/368, 435/455, 435/462, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 5837510 A

L11: Entry 3 of 4 File: USPT Nov 17, 1998

US-PAT-NO: 5837510
DOCUMENT-IDENTIFIER: US 5837 A

TITLE: Methods and polynucleotide constructs for treating host cells for infection or hyperproliferative disorders

DATE-ISSUED: November 17, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goldsmith; Mark A.	San Francisco	CA	94131	
Ralston; Robert O.	San Francisco	CA	94122	

US-CL-CURRENT: 435/455; 424/93.2, 435/320.1, 435/456, 514/44, 536/23.1, 536/23.2, 536/23.5, 536/23.53, 536/23.6, 536/23.7, 536/23.72, 536/24.1, 536/24.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

☐ 4. Document ID: US 5175103 A

L11: Entry 4 of 4 File: USPT Dec 29, 1992

US-PAT-NO: 5175103
DOCUMENT-IDENTIFIER: US 5175103 A

TITLE: Preparation of pure cultures of post-mitotic human neurons

DATE-ISSUED: December 29, 1992

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lee; Virginia	Philadelphia	PA		
Pleasure; Samuel	Philadelphia	PA		

US-CL-CURRENT: 435/455; 435/377

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

Generate Collection

Terms	Documents
l9 and ((435/455)!.CCLS.)	4

Display 20 Documents, starting with Document: 4

Display Format: Change Format

WEST

Generate Collection

Search Results - Record(s) 1 through 14 of 14 returned.

1. Document ID: US 20010033834 A1

L10: Entry 1 of 14 File: PGPB Oct 25, 2001

PGPUB-DOCUMENT-NUMBER: 20010033834
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20010033834 A1

TITLE: Pleuripotent stem cells generated from adipose tissue-derived stromal cells and uses thereof

PUBLICATION-DATE: October 25, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wilkison, William O.	Bahama	NC	US	
Gimble, Jeffrey	Chapel Hill	NC	US	

US-CL-CURRENT: 424/93.7; 424/93.21, 435/325, 435/366, 435/368, 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

2. Document ID: US 6225048 B1

L10: Entry 2 of 14 File: USPT May 1, 2001

US-PAT-NO: 6225048
DOCUMENT-IDENTIFIER: US 6225048 B1

TITLE: Dendritic-marker expressing macrophage cultures and methods of reactivating latent virus

DATE-ISSUED: May 1, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Soderberg-Naucler; Cecilia E.	Hagarsten			SEX
Fish; Kenneth N.	San Diego	CA		
Moses; Ashlee	Portland	OR		
Streblow; Daniel	Tigard	OR		
Nelson; Jay	Tualatin	OR		

US-CL-CURRENT: 435/5; 435/235.1, 435/325, 435/373

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

3. Document ID: US 6001654 A

L10: Entry 3 of 14 File: USPT Dec 14, 1999

US-PAT-NO: 6001654
DOCUMENT-IDENTIFIER: US 6001 A

TITLE: Methods for differentiating neural stem cells to neurons or smooth muscle cells using TGT-.beta. super family growth factors

DATE-ISSUED: December 14, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Shah; Nirao M.	New York	NY		

US-CL-CURRENT: 435/377; 435/325, 435/352, 435/353, 435/368, 435/375

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

4. Document ID: US 6001606 A

L10: Entry 4 of 14 File: USPT Dec 14, 1999

US-PAT-NO: 6001606
DOCUMENT-IDENTIFIER: US 6001606 A

TITLE: Polynucleotides encoding myeloid progenitor inhibitory factor-1 (MPIF-1) and polypeptides encoded thereby

DATE-ISSUED: December 14, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Ruben; Steven M.	Olney	MD		
Li; Haodong	Gaithersburg	MD		

US-CL-CURRENT: 435/69.5; 424/85.1, 435/252.3, 435/254.11, 435/320.1, 435/325, 435/471, 435/71.2, 536/23.1, 536/23.5, 930/140

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

5. Document ID: US 5928947 A

L10: Entry 5 of 14 File: USPT Jul 27, 1999

US-PAT-NO: 5928947
DOCUMENT-IDENTIFIER: US 5928947 A

TITLE: Mammalian multipotent neural stem cells

DATE-ISSUED: July 27, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/455; 424/93.7, 435/325, 435/440, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 6. Document ID: US 5919702 A

L10: Entry 6 of 14 File: USPT Jul 6, 1999

US-PAT-NO: 5919702
DOCUMENT-IDENTIFIER: US 5919702 A

TITLE: Production of cartilage tissue using cells isolated from Wharton's jelly

DATE-ISSUED: July 6, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Purchio; Anthony F.	La Jolla	CA		
Naughton; Brian A.	El Cajon	CA		
San Roman; Julia	San Diego	CA		

US-CL-CURRENT: 435/378; 424/93.1, 435/325, 435/366, 435/377

Full | Title | Citation | Front | Review | Classification | Date | Reference KWIC | Draw Desc | Image

---

☐ 7. Document ID: US 5888814 A

L10: Entry 7 of 14 File: USPT Mar 30, 1999

US-PAT-NO: 5888814
DOCUMENT-IDENTIFIER: US 5888814 A

TITLE: Recombinant host cells encoding TNF proteins

DATE-ISSUED: March 30, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kriegler; Michael	San Francisco	CA		
Perez; Carl F.	Berkeley	CA		

US-CL-CURRENT: 435/360; 424/93.1, 424/93.21, 424/93.7, 435/252.3, 435/320.1, 435/325, 435/363, 435/366, 435/371, 435/372 , 435/372.1, 435/69.5, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference KWIC | Draw Desc | Image

---

☐ 8. Document ID: US 5885829 A

L10: Entry 8 of 14 File: USPT Mar 23, 1999

US-PAT-NO: 5885829
DOCUMENT-IDENTIFIER: US 5885829 A

TITLE: Engineering oral tissues

DATE-ISSUED: March 23, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mooney; David J.	Ann Arbor	MI		
Rutherford; Robert B.	Ann Arbor	MI		

US-CL-CURRENT: 435/325; 424/422, 424/435, 424/49, 435/374, 435/378, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 9. Document ID: US 5858721 A

L10: Entry 9 of 14 File: USPT Jan 12, 1999

US-PAT-NO: 5858721
DOCUMENT-IDENTIFIER: US 5858721 A

TITLE: Three-dimensional cell and tissue culture system

DATE-ISSUED: January 12, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 435/69.1; 435/1.1, 435/320.1, 435/325, 435/326, 435/347, 435/363, 435/365.1, 435/366, 435/367, 435/368, 435/369, 435/370, 435/371, 435/372, 435/373, 435/396, 435/397, 435/398, 435/399, 435/402, 435/69.2, 435/69.3, 435/69.4, 435/69.5, 435/69.6, 435/70.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 10. Document ID: US 5849553 A

L10: Entry 10 of 14 File: USPT Dec 15, 1998

US-PAT-NO: 5849553
DOCUMENT-IDENTIFIER: US 5849553 A

TITLE: Mammalian multipotent neural stem cells

DATE-ISSUED: December 15, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/467; 435/320.1, 435/325, 435/353, 435/368, 435/455, 435/462, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

☐ 11. Document ID: US 5785964 A

L10: Entry 11 of 14 File: USPT Jul 28, 1998

US-PAT-NO: 5785964
DOCUMENT-IDENTIFIER: US 5785 A

TITLE: Three-dimensional genetically engineered cell and tissue culture system

DATE-ISSUED: July 28, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Naughton; Gail K.	Groton	VT		
Naughton; Brian A.	Groton	VT		

US-CL-CURRENT: 424/93.21; 424/93.1, 424/93.2, 424/93.3, 435/320.1, 435/325, 800/14

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

12. Document ID: US 5672499 A

L10: Entry 12 of 14 File: USPT Sep 30, 1997

US-PAT-NO: 5672499
DOCUMENT-IDENTIFIER: US 5672499 A

TITLE: Immoralized neural crest stem cells and methods of making

DATE-ISSUED: September 30, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/353; 435/320.1, 435/325, 435/368, 435/467, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

13. Document ID: US 5654183 A

L10: Entry 13 of 14 File: USPT Aug 5, 1997

US-PAT-NO: 5654183
DOCUMENT-IDENTIFIER: US 5654183 A

TITLE: Genetically engineered mammalian neural crest stem cells

DATE-ISSUED: August 5, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Anderson; David J.	Altadena	CA		
Stemple; Derek L.	Newton	MA		

US-CL-CURRENT: 435/456; 435/320.1, 435/325, 435/353, 435/368, 435/69.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | KWIC | Draw Desc | Image

---

14. Document ID: US 5604116 A

US-PAT-NO: 5604116
DOCUMENT-IDENTIFIER: US 5604116 A

TITLE: Interleukin-3 (IL-3) multiple mutation polypeptides, recombinant production of the same, and corresponding therapeutic methods

DATE-ISSUED: February 18, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bauer; S. Christopher	New Haven	MO		
Abrams; Mark A.	St. Louis	MO		
Braford-Goldberg; Sarah R.	St. Louis	MO		
Caparon; Maire H.	Chesterfield	MO		
Easton; Alan M.	Maryland Heights	MO		
Klein; Barbara K.	Town & Country	MO		
McKearn; John P.	Glencoe	MO		
Olins; Peter	Glencoe	MO		
Paik; Kumnan	Ballwin	MO		
Thomas; John W.	Town & Country	MO		

US-CL-CURRENT: 435/69.52; 424/85.2, 435/252.3, 435/254.11, 435/320.1, 435/325, 530/351, 536/23.5

```
=> d his

     (FILE 'HOME' ENTERED AT 17:15:34 ON 21 NOV 2001)

     FILE 'MEDLINE, BIOSIS, SCISEARCH, CAPLUS' ENTERED AT 17:15:59 ON 21 NOV
     2001
L1      1627605 S EMBRYONIC(W)(STEM OR GERM OR CARCINOMA) OR ES OR EG OR EC
L2         6904 S (FETAL OR POST-NATAL OR ADULT)(W)CELL
L3         2806 S (NEURAL OR NEURONAL)(6A)PROGENITOR
L4      1636707 S L1 OR L2 OR L3
L5         2942 S MULTIPOTENTIAL(5A)CELL
L6       533184 S SELECTABLE(5A)MARKER OR ANTIBIOTIC?
L7         7898 S SOX OR SOX1 OR SOX2 OR SOX3
L8       541050 S L6 OR L7
L9        21124 S L4 AND L8
L10          12 S L5 AND L8
L11         430 S L9 AND DIFFERENTIAT?
L12         164 S L11 AND CULTUR?
L13          14 S L12 AND LINEAGE
L14           8 DUP REM L10 (4 DUPLICATES REMOVED)
L15          11 DUP REM L13 (3 DUPLICATES REMOVED)

=> d au ti so ab 1-8 l14

L14  ANSWER 1 OF 8     MEDLINE                                  DUPLICATE 1
AU   Nishimura Ji; Phillips K L; Ware R E; Hall S; Wilson L; Gentry T L;
Howard
     T A; Murakami Y; Shibano M; Machii T; Gilboa E; Kanakura Y; Takeda J;
     Kinoshita T; Rosse W F; Smith C A
TI   Efficient retrovirus-mediated PIG-A gene transfer and stable restoration
     of GPI-anchored protein expression in cells with the PNH phenotype.
SO   BLOOD, (2001 May 15) 97 (10) 3004-10.
     Journal code: A8G; 7603509. ISSN: 0006-4971.
AB   Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem
     cell disorder characterized by complement-mediated hemolysis due to
     deficiencies of glycosylphosphatidylinositol-anchored proteins (GPI-APs)
     in subpopulations of blood cells. Acquired mutations in the X-linked
     phosphatidylinositol glycan-class A (PIG-A) gene appear to be the
     characteristic and pathogenetic cause of PNH. To develop a gene therapy
     approach for PNH, a retroviral vector construct, termed MPIN, was made
     containing the PIG-A complementary DNA along with an internal ribosome
     entry site and the nerve growth factor receptor (NGFR) as a
     selectable marker. MPIN transduction led to efficient
     and stable PIG-A and NGFR gene expression in a PIG-A-deficient B-cell
line
     (JY5), a PIG-A-deficient K562 cell line, an Epstein-Barr
virus-transformed
     B-cell line (TK-14(-)) established from a patient with PNH, as well as
     peripheral blood (PB) mononuclear cells from a patient with PNH. PIG-A
     expression in these cell lines stably restored GPI-AP expression. MPIN
was
     transduced into bone marrow mononuclear cells from a patient with PNH,
and
     myeloid/erythroid colonies and erythroid cells were derived. These
     transduced erythroid cells restored surface expression of GPI-APs and
     resistance to hemolysis. These results indicate that MPIN is capable of
     efficient and stable functional restoration of GPI-APs in a variety of
     PIG-A-deficient hematopoietic cell types. Furthermore, MPIN also
     transduced into PB CD34(+) cells from a normal donor, indicating that
MPIN
```

can transduce primitive human progenitors. These findings set the stage for determining whether MPIN can restore PIG-A function in **multipotential** stem **cells**, thereby providing a potential new therapeutic option in PNH.

L14 ANSWER 2 OF 8 MEDLINE
AU Manabe I; Owens G K
TI Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during differentiation of a novel P19-derived in vitro smooth muscle differentiation system.
SO CIRCULATION RESEARCH, (2001 Jun 8) 88 (11) 1127-34.
Journal code: DAJ; 0047103. ISSN: 1524-4571.
AB Little is known regarding transcriptional regulatory mechanisms that control the sequential and coordinate expression of genes during smooth muscle cell (SMC) differentiation. To facilitate mechanistic studies of SMC differentiation, we established a novel P19-derived clonal cell line (designated A404) harboring a smooth muscle (SM) alpha-actin promoter/intron-driven puromycin resistance gene. Retinoic acid plus puromycin treatment stimulated rapid differentiation of **multipotential** A404 **cells** into SMCs that expressed multiple SMC differentiation marker genes, including the definitive SM-lineage marker SM myosin heavy chain. Using this system, we demonstrated that various transcription factors were upregulated coincidentally with the expression of SMC differentiation marker genes. Of interest, the expression of serum response factor (SRF), whose function is critical for SMC-specific transcription, was high in undifferentiated A404 cells, and it did not increase over the course of differentiation. However, chromatin immunoprecipitation analyses showed that SRF did not bind the target sites of endogenous SMC marker genes in chromatin in undifferentiated cells, but it did in differentiated A404 cells, and it was associated with hyperacetylation of histones H3 and H4. The present studies define a novel cell system for studies of transcriptional regulation during the early stages of SMC differentiation, and using this system, we obtained evidence for the involvement of chromatin remodeling and selective recruitment of SRF to CArG elements in the induction of cell-selective marker genes during SMC differentiation.

L14 ANSWER 3 OF 8 CAPLUS COPYRIGHT 2001 ACS
AU Majumdar, Manas Kumar; Wang, Eunice; Morris, Elisabeth Ann
TI BMP-2 and BMP-9 promote chondrogenic differentiation of human **multipotential** mesenchymal **cells** and overcome the inhibitory effect of IL-1
SO J. Cell. Physiol. (2001), 189(3), 275-284
CODEN: JCLLAX; ISSN: 0021-9541
AB Bone morphogenetic proteins play important roles in connective tissue morphogenesis. In this study, we used human **multipotential** mesenchymal **cells** as a target to analyze the effect of bone morphogenetic proteins on chondrogenesis. We also analyzed the effect of proinflammatory cytokine interleukin-1 on chondrogenic-differentiated cells and the interaction of IL-1.beta. with bone morphogenetic proteins. Cells placed in a 3-dimensional matrix of alginate beads and cultured in a serum-free media with bone morphogenetic protein-2 and -9 induced expression of type II collagen (Col2A1) mRNA and increased expression of aggrecan and cartilage oligomeric matrix protein suggesting chondrogenic differentiation of the cells. The transcription factor **Sox**-9 that regulates both Col2A1 and aggrecan gene expression showed increased

expression with BMP treatment. Chondrogenic differentiated cells treated with interleukin-1 decreased **Sox**-9, Col2A1 and aggrecan gene expression. Removal of interleukin-1 and further addn. of bone morphogenetic proteins resulted in returned expression of chondrogenic markers. Chondrogenic differentiated cells cultured in the presence of different concns. of bone morphogenetic proteins and interleukin-1 showed that bone morphogenetic proteins were able to partially block the suppressive effect of interleukin-1. This study shows that bone morphogenetic proteins play an important role in chondrogenesis and may prove to be potential therapeutics in cartilage repair.

L14 ANSWER 4 OF 8 BIOSIS COPYRIGHT 2001 BIOSIS
AU Li, M. (1); Smith, A. (1)
TI Derivation of **multipotential** neural precursors from embryonic stem **cells** in vitro.
SO European Journal of Neuroscience, (2000) Vol. 12, No. Supplement 11, pp. 324. print.
Meeting Info.: Meeting of the Federation of European Neuroscience Societies Brighton, UK June 24-28, 2000
ISSN: 0953-816X.

L14 ANSWER 5 OF 8 CAPLUS COPYRIGHT 2001 ACS
IN Smith, Austin; Li, Meng
TI Lineage specific cells and progenitor cells
SO PCT Int. Appl.
CODEN: PIXXD2
AB A method for generating a culture that is purified or enriched in respect of cells of a selected lineage is described in which a **selectable marker**, which is differentially expressed in cells of the selected lineage compared with its expression in other **cells**, is introduced into a **multipotential cell** and the **multipotential cell** is cultured to induce differentiation of the **multipotential cell** into a **cell** of the selected lineage or into a mixture of cells including cells of the selected lineage, or is cultured to induce preferential survival of cells of the selected lineage. Those cells that express the **selectable marker** are then selected for. Progenitors of selected lineage are also described as is the use of the method in assay techniques.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31
Journal code: BNQ; 9007422. ISSN: 1042-8194.
AB Autologous- or allogeneic-bone marrow transplantation are increasingly used to overcome the myelosuppressive effects of high dose chemotherapy administered to cancer patients. Transfer of the multidrug resistance (MDR) gene in hemopoietic progenitors has been proposed as a tool to administer higher and possibly more curative doses of chemotherapy. Murine models have demonstrated that retrovirus-mediated MDR transfer in bone marrow cells can render animals resistant to myeloablative doses of Taxol, and in vitro studies have shown that MDR-transduced human CD34+ **cells** can generate drug-resistant **multipotential** hemopoietic progenitors such as long term culture-initiating cells. Given these results, phase I clinical trials are currently under way to evaluate

feasibility and treatment-related toxicity of MDR gene transfer in cancer patients by means of safe retroviral vectors. Finally, Taxol treatment of MDR transduced mice and human CD34+ cells have indicated that MDR is a dominant **selectable marker** in vitro and in vivo, and vectors carrying both MDR and non selectable genes such as beta-globin or glucocerebrosidase could be used in the next future for gene therapy of inherited disorders like thalassemia or Gaucher disease.

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.
ISSN: 1042-8194.
AB Autologous- or allogeneic-bone marrow transplantation are increasingly used to overcome the myelosuppressive effects of high dose chemotherapy administered to cancer patients. Transfer of the multidrug resistance (MDR) gene in hemopoietic progenitors has been proposed as a tool to administer higher and possibly more curative doses of chemotherapy. Murine models have demonstrated that retrovirus-mediated MDR transfer in bone marrow cells can render animals resistant to myeloablative doses of Taxol, and in vitro studies have shown that MDR-transduced human CD34+ **cells** can generate drug-resistant **multipotential** hemopoietic progenitors such as long term culture-initiating cells. Given these results, phase I clinical trials are currently under way to evaluate feasibility and treatment-related toxicity of MDR gene transfer in cancer patients by means of safe retroviral vectors. Finally, Taxol treatment of MDR transduced mice and human CD34+ cells have indicated that MDR is a dominant **selectable marker** in vitro and in vivo, and vectors carrying both MDR and non selectable genes such as beta-globin or glucocerebrosidase could be used in the next future for gene therapy of inherited disorders like thalassemia or Gaucher disease.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.
Journal code: IYG; 7505566. ISSN: 0025-7850.
AB To study the ability of endosteal cells to spontaneously differentiate in vitro, cells isolated from the endosteal bone surface (endosteal cells) were incubated in liquid suspension cultures containing fetal calf serum and **antibiotics**, but without chemical inducers of differentiation. Cultures were examined daily for the appearance of morphologically recognizable mature and differentiated hemopoietic cells. Culture outcomes were uniformly consistent, showed an almost complete disappearance of the endosteal cells (which appeared as stimulated lymphoid and late stage normoblast-like cells once they are separated from the bone surfaces), and their replacement by differentiated and mature hemopoietic cells. The ability of endosteal cells to fully differentiate into mature hemopoietic cells in vitro indicates that endosteal **cells** represent the **multipotential** hemopoietic stem **cells** in vivo, and are equivalent of embryonal stage undifferentiated mesenchymal cells.

=> d bib 5 l14

L14 ANSWER 5 OF 8 CAPLUS COPYRIGHT 2001 ACS
AN 1999:670196 CAPLUS
TI Lineage specific cells and progenitor cells
IN Smith, Austin; Li, Meng
PA University of Edinburgh, UK
SO PCT Int. Appl.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9953022	A2	19991021	WO 1999-GB1136	19990414
	WO 9953022	A3	20010419		
	W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	RW: GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
	US 6146888	A	20001114	US 1995-535141	19951229
	AU 9934357	A1	19991101	AU 1999-34357	19990414
	EP 1115840	A2	20010718	EP 1999-915938	19990414
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
PRAI	US 1995-535141	A2	19951229		
	GB 1998-7935	A	19980414		
	GB 1993-8271	A	19930421		
	WO 1994-GB848	W	19940421		
	WO 1999-GB1136	W	19990414		

=> d 1-11 au ti so l15

L15 ANSWER 1 OF 11 MEDLINE
AU Franceschini I A; Feigenbaum-Lacombe V; Casanova P; Lopez-Lastra M; Darlix J L; Dalcq M D
TI Efficient gene transfer in mouse neural precursors with a bicistronic retroviral vector.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (2001 Aug 1) 65 (3) 208-19.
Journal code: KAC; 7600111. ISSN: 0360-4012.

L15 ANSWER 2 OF 11 MEDLINE
AU Chandross K J; Cohen R I; Paras P Jr; Gravel M; Braun P E; Hudson L D
TI Identification and characterization of early glial progenitors using a transgenic selection strategy.
SO JOURNAL OF NEUROSCIENCE, (1999 Jan 15) 19 (2) 759-74.
Journal code: JDF; 8102140. ISSN: 0270-6474.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.

SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 4 OF 11 MEDLINE
AU Morceau F; Aries A; Lahlil R; Devy L; Jardillier J C; Jeannesson P; Trentesaux C
TI Evidence for distinct regulation processes in the aclacinomycin- and doxorubicin-mediated **differentiation** of human erythroleukemic cells.
SO BIOCHEMICAL PHARMACOLOGY, (1996 Mar 22) 51 (6) 839-45.
Journal code: 9Z4; 0101032. ISSN: 0006-2952.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of **differentiated** cells permits isolation of **embryonic stem** cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from **differentiating** embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of **differentiation** and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from **embryonic stem** cells in **culture**.
SO SCIENCE, (1994 Aug 19) 265 (5175) 1098-101.
Journal code: UJ7; 0404511. ISSN: 0036-8075.

L15 ANSWER 9 OF 11 MEDLINE
AU Francis G E; Tejedor M C; Berney J J; Chresta C M; Delgado C; Patel P
TI Effects of DNA topoisomerase II inhibitors on human bone marrow progenitor cells.
SO LEUKEMIA, (1994 Jan) 8 (1) 121-8.
Journal code: LEU; 8704895. ISSN: 0887-6924.

L15 ANSWER 10 OF 11 MEDLINE
AU Okado H; Takahashi K
TI Neural **differentiation** in cleavage-arrested ascidian blastomeres induced by a proteolytic enzyme.
SO JOURNAL OF PHYSIOLOGY, (1993 Apr) 463 269-90.
Journal code: JQV; 0266262. ISSN: 0022-3751.

L15 ANSWER 11 OF 11 MEDLINE
AU Klein B Y; Gal I; Segal D
TI Selection of malonate-resistant stromal cell-derived osteoprogenitor cells
in vitro.
SO JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 190-7.
Journal code: HNF; 8205768. ISSN: 0730-2312.

=>

=> d 1-11 au ti so ab l15

L15 ANSWER 1 OF 11 MEDLINE
AU Franceschini I A; Feigenbaum-Lacombe V; Casanova P; Lopez-Lastra M; Darlix
J L; Dalcq M D
TI Efficient gene transfer in mouse neural precursors with a bicistronic retroviral vector.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (2001 Aug 1) 65 (3) 208-19.
Journal code: KAC; 7600111. ISSN: 0360-4012.
AB Gene transfer into neural precursors is a powerful approach to study the function of specific gene products during nervous system development. Here
we describe a retrovirus-based methodology to transduce foreign genes into
mouse neural precursors. We used a high-titer bicistronic retroviral vector that encodes a marker gene, placental alkaline phosphatase (plap), and a selection gene, neomycin phosphotransferase II (neoR), under the translational control of two retroviral internal ribosome entry segments. Transduction efficiency even without selection was up to 95% for multipotential neurospheres derived from embryonic striata and grown with basic fibroblast growth factor 2. Expression of plap and neoR was sustained with time in **culture** and upon **differentiation** into neurons, astrocytes, and oligodendrocytes, as shown by double immunofluorescence labeling with cell type-specific markers, Western blotting, and neomycin resistance. However, levels of plap were decreased in **differentiated** oligodendrocytes. Transduction with the same vector of neonatal oligodendrocyte precursors grown in oligospheres consistently resulted in a lower proportion of plap-immunoreactive cells and enhanced cell death in the absence of neomycin. However, plap expression was maintained in some **differentiated** oligodendrocytes expressing galactocerebroside or myelin basic protein. In
that neurospheres can be easily expanded in vitro and factors enabling their **differentiation** into the three main central nervous system cell types are being elucidated, this methodology could be used in the future to produce large number of transduced, **differentiated** neural cells.

L15 ANSWER 2 OF 11 MEDLINE
AU Chandross K J; Cohen R I; Paras P Jr; Gravel M; Braun P E; Hudson L D
TI Identification and characterization of early glial progenitors using a transgenic selection strategy.
SO JOURNAL OF NEUROSCIENCE, (1999 Jan 15) 19 (2) 759-74.
Journal code: JDF; 8102140. ISSN: 0270-6474.
AB To define the spatiotemporal development of and simultaneously select for oligodendrocytes (OLs) and Schwann cells (SCs), transgenic mice were generated that expressed a bacterial beta-galactosidase (beta-gal) and neomycin phosphotransferase fusion protein (betageo) under the control of murine 2'3'-cyclic nucleotide 3'-phosphodiesterase (muCNP) promoters I and
II. Transgenic beta-gal activity was detected at embryonic day 12.5 in the
ventral region of the rhombencephalon and spinal cord and in the neural crest. When cells from the rhombencephalon were **cultured** in the presence of G418, surviving cells **differentiated** into OLs, indicating that during development this brain region provides one source of OL progenitors. Postnatally, robust beta-gal activity was localized to

OLs throughout the brain and was absent from astrocytes, neurons, and microglia or monocytes. In the sciatic nerve beta-gal activity was localized exclusively to SCs. **Cultures** from postnatal day 10 brain or sciatic nerve were grown in the presence of G418, and within 8-9 d exposure to **antibiotic**, 99% of all surviving cells were beta-gal-positive OLs or SCs. These studies demonstrate that the muCNP-betageo transgenic mice are useful for identifying OLs and SCs beginning at early stages of the glial cell **lineage** and throughout their development. This novel approach definitively establishes that the beta-gal-positive cells identified in vivo are glial progenitors, as defined by their ability to survive **antibiotic** selection and **differentiate** into OLs or SCs in vitro. Moreover, this experimental paradigm facilitates the rapid and efficient selection of pure populations of mouse OLs and SCs and further underscores the use of cell-specific promoters in the purification of distinct cell types.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.
AB Delivery of genes into hematopoietic progenitor cells offers an attractive means for the introduction of corrective or protective genes into cells of both the myeloid and lymphoid **lineage**. Previously, investigators have often used murine retroviral vectors for gene delivery which require cells to be cycling for efficient delivery. We describe a nonviral method of gene delivery using particle-mediated gene transfer to obviate many disadvantages of viral vectors related to safety, production costs and the need for cell cycle proliferation. Using a CMV-CAT reporter plasmid, we show transfection of highly purified CD34+ cells isolated from umbilical cord blood. Effective gene transfer was shown in unstimulated and in growth-stimulated cells. Following transfection with a neomycin resistance gene, **differentiation** into cells of the myeloid **lineage** was observed, assayed by CFU-GM in the presence of G-418. Both unstimulated and stimulated cells gave rise to CFU-GM in the presence of G-418, indicating that stable expression of the neomycin resistance gene was maintained in early progenitors. These results demonstrate that particle-mediated gene transfer into human hematopoietic cells from umbilical cord blood can be achieved without affecting their CFU-GM **differentiation** potential. This gene transfer method offers an alternative approach to gene therapy studies involving human hematopoietic progenitor cells.

L15 ANSWER 4 OF 11 MEDLINE
AU Morceau F; Aries A; Lahlil R; Devy L; Jardillier J C; Jeannesson P; Trentesaux C
TI Evidence for distinct regulation processes in the aclacinomycin- and doxorubicin-mediated **differentiation** of human erythroleukemic cells.
SO BIOCHEMICAL PHARMACOLOGY, (1996 Mar 22) 51 (6) 839-45.
Journal code: 9Z4; 0101032. ISSN: 0006-2952.

AB Human erythroleukemic K 562 cells were induced to were induced to **differentiate** along the erythroid **lineage** by anthracycline antitumor drugs, such as aclacinomycin (ACLA) and doxorubicin (DOX). Subsequent stimulation of heme and globin synthesis led to a differential quantitative expression of hemoglobins. Gower 1 (epsilon2, zeta2) was the major type for ACLA and X (epsilon2, gamma2) for DOX. Although ACLA and DOX increased both the expression of gamma-globin and porphobilinogen deaminase mRNAs, striking differences were observed in the expression of erythropoietin receptor mRNAs and in erythroid transcription factors GATA-1 and NF-E2, known to play a key role in erythroid gene regulation. Indeed, ACLA induces an increase either in the binding capacity of GATA-1 and NF-E2 or in the accumulation of erythropoietin receptor, GATA-1 and NF-E2 transcripts. In contrast, their expression with DOX was not significantly modified compared to uninduced cells, except for a slight decrease in NF-E2 expression on day 3. In conclusion, these data show that: 1. increased expression of erythroid transcription factors and erythroid genes are associated only with ACLA treatment, and 2. although cytotoxicity of both ACLA and DOX is certainly dependent on DNA intercalation, regulation of **differentiation** processes by these two drugs involves distinct mechanisms.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of **differentiated** cells permits isolation of **embryonic stem** cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.
AB **Embryonic stem** (**ES**) cells enable the engineering of precise modifications to the mouse genome by gene targeting. Although there are reports of **cultured** cell contributions to chimaeras in golden hamster, rat and pig, definitive **ES** cell lines which contribute to the germline have not been demonstrated in any species but mouse. Among mouse strains, genetic background strongly affects the efficiency of **ES** isolation, and almost all **ES** lines in use are derived from strain 129 (refs 1,4,5) or, less commonly, C57BL/6 (refs 6-8). The CBA strain is refractory to **ES** isolation and there are no published reports of CBA-derived **ES** lines. Hence, CBA mice may provide a convenient model of **ES** isolation in other species. In **ES** derivation it is critical that the primary explant be **cultured** for a sufficient time to allow multiplication of **ES** cell progenitors, yet without allowing extensive **differentiation**. Thus, differences in **ES** derivation between mouse strains may reflect differences in the control of **ES** progenitor cells by other **lineages** within the embryo. Here we describe a strategy to continuously remove **differentiated** cells by drug selection, which generates germline competent **ES** lines from genotypes that are non-permissive in the absence of selection.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from **differentiating** embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.

Journal code: HS7; 7802877. ISSN: 0021-9738.
AB This study describes a simple approach to generate relatively pure **cultures** of cardiomyocytes from **differentiating** murine **embryonic stem** (**ES**) cells. A fusion gene consisting of the alpha-cardiac myosin heavy chain promoter and a cDNA encoding aminoglycoside phosphotransferase was stably transfected into pluripotent **ES** cells. The resulting cell lines were **differentiated** in vitro and subjected to G418 selection. Immunocytological and ultrastructural analyses demonstrated that the selected cardiomyocyte **cultures** (> 99% pure) were highly **differentiated**. G418 selected cardiomyocytes were tested for their ability to form grafts in the hearts of adult dystrophic mice. The fate of the engrafted cells was monitored by antidystrophin immunohistology, as well as by PCR analysis with primers specific for the myosin heavy chain-aminoglycoside phosphotransferase transgene. Both analyses revealed the presence of **ES**-derived cardiomyocyte grafts for as long as 7 wk after implantation, the latest time point analyzed. These studies indicate that a simple genetic manipulation can be used to select essentially pure **cultures** of cardiomyocytes from **differentiating ES** cells. Moreover, the resulting cardiomyocytes are suitable for the formation of intracardiac grafts. This selection approach should be applicable to all **ES**-derived cell **lineages**.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of **differentiation** and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.
AB Cell sublines resistant to doxorubicin (DOX) were developed from the human leukemia cell line, U-937/WT, exposed to stepwise DOX increases. In contrast to U-937/WT cells, the DOX-resistant U-937/RD cells have longer doubling time; are more **differentiated** along the monocytic **lineage** as determined by the presence of morphological features and mRNA coding for the monocyte colony-stimulating factor-1 receptor; synthesize the apoptosis-associated Bax protein; are less sensitive to apoptosis-inducing topoisomerase II-directed drugs, apparently because of increased synthesis of P-glycoprotein; and are practically non-tumorigenic when xenografted in nude mice. However, U-937/WT and U-937/RD cells exhibit similar sensitivity to the apoptosis-inducing drug 9-nitrocamptothecin. These findings suggest that several mechanisms are involved in the development of DOX-resistance in U-937 cells, and further, 9-nitrocamptothecin can overcome resistance to DOX. These findings may have clinical implications.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from **embryonic stem** cells in **culture**.
SO SCIENCE, (1994 Aug 19) 265 (5175) 1098-101.
Journal code: UJ7; 0404511. ISSN: 0036-8075.

AB An efficient system was developed that induced the **differentiation** of **embryonic stem** (**ES**) cells into blood cells of erythroid, myeloid, and B cell **lineages** by coculture with the stromal cell line OP9. This cell line does not express functional macrophage colony-stimulating factor (M-CSF). The presence of M-CSF had inhibitory effects on the **differentiation** of **ES** cells to blood cells other than macrophages. Embryoid body formation or addition of exogenous growth factors was not required, and **differentiation** was highly reproducible even after the selection of **ES** cells with the **antibiotic** G418. Combined with the ability to genetically manipulate **ES** cells, this system will facilitate the study of molecular mechanisms involved in development and **differentiation** of hematopoietic cells.

L15 ANSWER 9 OF 11 MEDLINE
AU Francis G E; Tejedor M C; Berney J J; Chresta C M; Delgado C; Patel P
TI Effects of DNA topoisomerase II inhibitors on human bone marrow progenitor cells.
SO LEUKEMIA, (1994 Jan) 8 (1) 121-8.
Journal code: LEU; 8704895. ISSN: 0887-6924.
AB Topoisomerase II (topo II) is a target for many cytotoxic agents. Two observations, however, warrant caution in their therapeutic use: first, these agents can inhibit **differentiation** and second, perturbations in function render the enzyme error-prone. Illegitimate recombination events occurring at sites where topo II acts in **differentiation** could be particularly important in the development of secondary malignancies (relatively frequent after therapy with agents that target topo II). Topo II inhibitors are heterogeneous in mechanisms of action; in site-specificity of cleavable complex 'entrapment' (where present) and in the relative potency against the two topo II isoforms, all potentially influencing the site of maximum DNA damage. The object of this study was to examine the effect of topo II inhibitors on human haemopoietic precursor cells, to determine which have most impact on **differentiation**. We selected two which act via cleavable complex entrapment, but with different site preferences (m-AMSA and VP-16), and two acting via other mechanisms (merbarone and fostriecin). VP-16 and m-AMSA showed similar patterns with low dose stimulation of granulocyte-macrophage colony formation and high dose inhibition of all colony types. The stimulation was accompanied by an increase in colony size and blast content, consistent with a low dose inhibition of **differentiation**. Forstriecin, in contrast, stimulated predominantly mixed and erythroid colonies. Merbarone failed to increase colony formation. Neither produced substantial inhibition of colony formation. The effects on granulocyte-macrophage progenitors were confirmed using 7-day suspension **cultures**, using nitroblue tetrazolium (NBT) reduction and 3-4,5,dimethylthiazol 2,5-diphenyl tetrazolium bromide (MTT) assays for **differentiated** cells and total cell mass, respectively. These results demonstrate that the effects of topo II inhibitors on haemopoietic cell proliferation and **differentiation** are agent-specific and can involve **lineage** -restricted partial inhibition of **differentiation**.

L15 ANSWER 10 OF 11 MEDLINE
AU Okado H; Takahashi K
TI Neural **differentiation** in cleavage-arrested ascidian blastomeres

induced by a proteolytic enzyme.
SO JOURNAL OF PHYSIOLOGY, (1993 Apr) 463 269-90.
Journal code: JQV; 0266262. ISSN: 0022-3751.
AB 1. As previously reported, ectodermal a4-2 blastomeres isolated from 8-cell embryos of the ascidian, Halocynthia roretzi or aurantium, and **cultured** under conditions of cleavage arrest always **differentiated** into an epidermal phenotype, showing long-lasting Ca(2+)-dependent action potentials and/or tunic on the cell surface. a4-2 blastomeres contacted by a chordamesodermal blastomere, A4-1, **differentiated** into a neural phenotype, characterized by fast Na(+)-dependent spikes. **Differentiation** to a similar neural phenotype occurred when isolated a4-2 blastomeres from H. aurantium embryos were treated with > 0.003% subtilisin for 60 min at the 32-cell stage of the control embryo. Comparisons between induction by cell contact and induction by proteolytic enzymes were made and showed them to be similar in several respects. 2. When the serine protease, subtilisin, was used as the neural inducer, neural competence of a4-2 blastomeres, measured as the percentage frequency of the induction of Na+ spikes, increased after the 32-cell stage and decreased during the gastrula stage. The time course of the neural competence was the same as that for contact with the A4-1 blastomere. 3. The neural competence of four different ectodermal blastomeres isolated from the 16-cell embryo was also examined using subtilisin as a neural inducer, and by contact with the A4-1 blastomere from the 8-cell embryo. The competence was higher in anterior blastomeres than in posterior blastomeres for both types of induction. This regional difference in neural competence along the antero-posterior axis paralleled that expected from neural cell **lineage** during normal development, i.e. blastomeres with more cells of neural **lineage** among their derivatives showed higher competence. 4. Streptomyces subtilisin inhibitor, SSI (0.1%), a specific protease inhibitor for subtilisin-type serine proteases, significantly suppressed (50%) neural induction of the ectodermal blastomere, a4-2, by contact with the chordamesodermal blastomere, A4-1. 5. Monensin, brefeldin A and bafilomycin A1, all of which affect secretory processes, suppressed the neural inducing ability of the chordamesodermal blastomere, A4-1. 6. These results permit the hypothesis that a protease secreted from the chordamesoderm-generating blastomere induces the ectodermal blastomere to **differentiate** into neural cell type.

L15 ANSWER 11 OF 11 MEDLINE
AU Klein B Y; Gal I; Segal D
TI Selection of malonate-resistant stromal cell-derived osteoprogenitor cells in vitro.
SO JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 190-7.
Journal code: HNF; 8205768. ISSN: 0730-2312.
AB Bone marrow stromal cells give rise to osteoprogenitor cell (OPC) colonies, with characteristic mineralized bone nodules in vitro. During **differentiation**, OPCs in the **culture** are surrounded by heterogeneous populations of various cell **lineages** and by different OPC **differentiation** stages. In the present study, attempts were made to increase the homogeneity of OPCs in **culture**. The reliance on energy metabolism restricted to glycolysis, which is specific to the premineralizing skeletal cells, was tested as a **selectable marker** for cells in this stage. Day 12 alkaline phosphatase (ALP) and day 20-21 calcium precipitates were used as

early and late OPC **differentiation** markers. Malonate, a competitive inhibitor of succinate dehydrogenase, was added to the OPC stimulation medium, to interfere with the Krebs cycle-dependent energy metabolism operating in most of the stromal cells. OPCs that entered the stage of energy metabolism restricted to glycolysis were expected to become malonate resistant. Malonate showed dose and time dependence, 10 mM malonate added on day 3, decreased day 12 ALP activity/well to the lowest level. Variations in time and length of exposure to malonate used during the first 12 days of **differentiation** showed an inverse correlation between specific ALP activity and cell yield. Malonate-treated variations of specific ALP and of cell yield indices were up to 30- to 40-fold larger than variations within day 21 calcium precipitates. Thus, calcifying cells were almost unchanged relatively to noncalcifying cells. These results indicate that malonate-resistant cells are mostly selected, rather than induced, to **differentiate** by malonate. The results also show that stromal derived OPCs undergo a similar biochemical stage as in chondrocytes.

=>

stem cells in culture.
SO SCIENCE, (1994 Aug 19) 265 (5175) 1098-101.
Journal code: UJ7; 0404511. ISSN: 0036-8075.

5093055 Appl. J95 mios

L15 ANSWER 10 OF 11 MEDLINE
AU Okado H; Takahashi K
TI Neural differentiation in cleavage-arrested ascidian blastomeres induced by a proteolytic enzyme.
SO JOURNAL OF PHYSIOLOGY, (1993 Apr) 463 269-90.
Journal code: JQV; 0266262. ISSN: 0022-3751.

372864

L15 ANSWER 11 OF 11 MEDLINE
AU Klein B Y; Gal I; Segal D
TI Selection of malonate-resistant stromal cell-derived osteoprogenitor cells in vitro.
SO JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 190-7.
Journal code: HNF; 8205768. ISSN: 0730-2312.

824183

Shin-Lin Chen
AU 1633
CM1 12A15
Mail Box # 12E12
(703)305-1678

11/23
09688880

Journal of Physiology (1993), **463**, *pp.* 269 290
With 8 figures
Printed in Great Britain

NEURAL DIFFERENTIATION IN CLEAVAGE-ARRESTED ASCIDIAN BLASTOMERES INDUCED BY A PROTEOLYTIC ENZYME

BY HARUO OKADO AND KUNITARO TAKAHASHI

From the Department of Neurobiology, Institute for Brain Research, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

(*Received 18 December 1991*)

SUMMARY

1. As previously reported, ectodermal $a_{4\cdot2}$ blastomeres isolated from 8-cell embryos of the ascidian, *Halocynthia roretzi* or *aurantium*, and cultured under conditions of cleavage arrest always differentiated into an epidermal phenotype, showing long-lasting Ca^{2+}-dependent action potentials and/or tunic on the cell surface. $a_{4\cdot2}$ blastomeres contacted by a chordamesodermal blastomere, $A_{4\cdot1}$, differentiated into a neural phenotype, characterized by fast Na^+-dependent spikes. Differentiation to a similar neural phenotype occurred when isolated $a_{4\cdot2}$ blastomeres from *H. aurantium* embryos were treated with > 0·003 % subtilisin for 60 min at the 32-cell stage of the control embryo. Comparisons between induction by cell contact and induction by proteolytic enzymes were made and showed them to be similar in several respects.

2. When the serine protease, subtilisin, was used as the neural inducer, neural competence of $a_{4\cdot2}$ blastomeres, measured as the percentage frequency of the induction of Na^+ spikes, increased after the 32-cell stage and decreased during the gastrula stage. The time course of the neural competence was the same as that for contact with the $A_{4\cdot1}$ blastomere.

3. The neural competence of four different ectodermal blastomeres isolated from the 16-cell embryo was also examined using subtilisin as a neural inducer, and by contact with the $A_{4\cdot1}$ blastomere from the 8-cell embryo. The competence was higher in anterior blastomeres than in posterior blastomeres for both types of induction. This regional difference in neural competence along the antero-posterior axis paralleled that expected from neural cell lineage during normal development, i.e. blastomeres with more cells of neural lineage among their derivatives showed higher competence.

4. *Streptomyces* subtilisin inhibitor, SSI (0·1 %), a specific protease inhibitor for subtilisin-type serine proteases, significantly suppressed (50 %) neural induction of the ectodermal blastomere, $a_{4\cdot2}$, by contact wtih the chordamesodermal blastomere, $A_{4\cdot1}$.

5. Monensin, brefeldin A and bafilomycin A1, all of which affect secretory processes, suppressed the neural inducing ability of the chordamesodermal blastomere, $A_{4\cdot1}$.

6. These results permit the hypothesis that a protease secreted from the

MS 9984

chordamesoderm-generating blastomere induces the ectodermal blastomere to differentiate into neural cell type.

INTRODUCTION

A simple system of neural induction has been reconstituted using two cleavage-arrested blastomeres isolated from ascidian-8-cell embryos. The blastomeres of ascidian embryos are known to differentiate even in the cleavage-arrested condition (Whittaker, 1973). The anterior-animal or ectodermal blastomere, $a_{4.2}$, which gives rise to both epidermal and neural tissues in the normal development (Conklin, 1905; Nishida, 1987), generates sharp Na^+ spikes or long-lasting Ca^{2+} action potentials, following neural or epidermal differentiation respectively, in cleavage-arrested 8-cell embryos of *Halocynthiae* (Hirano, Takahashi & Yamashita, 1984). When the blastomere was isolated at the 8-cell stage and cultured under cleavage-arrested conditions, it was always found to differentiate into an epidermal phenotype when examined electrophysiologically and immunohistochemically, whereas, when it was cultured in contact with an anterior-vegetal chordamesodermal blastomere, $A_{4\cdot1}$, it differentiated almost exclusively into a neural phenotype (Okado & Takahashi, 1988; 1990*a*, *b*, summarized in Fig. 8 of *b*). This phenomenon is comparable to neural induction, which has been studied for a long time in the early development of amphibian (Spemann, 1938; Gurdon, 1987; Hamburger, 1988) and ascidian embryos (Rose 1939; Reverberi, 1971; Whittaker, 1987; Nishida & Satoh, 1989). Recently, in addition, we found that pronase, a mixture of proteolytic enzymes, has neural inducing activity in the cleavage-arrested ascidian blastomere (Okado & Takahashi, 1990*b*).

There is some question as to whether the inducing activity of proteolytic enzymes is an essential process during induction by contact. To answer this, we have carried out three kinds of experiments. First, we tried to identify some purified proteases which have the same inducing activity as pronase, since pronase is a mixture of multiple proteases and therefore complicates analysis of the inductive processes involved in neural differentiation. We show here that a purified serine protease, subtilisin, has potent inducing activity. Second, since subtilisin in combination with its specific inhibitor, *Streptomyces* subtilisin inhibitor, provided us with a method of applying the inducer at precise times, the time sequence of changes in neural competence induced by subtilisin was compared with that induced by cell contact. Third, the regional difference of neural competence induced by subtilisin in four different ectodermal blastomeres from the 16-cell embryo was determined and compared with that induced by contact with $A_{4\cdot1}$.

In addition, we have examined whether any protease inhibitors interfere with neural induction by cell contact, and whether substances that affect secretion, such as monensin, suppress induction. We suggest the inducer cell secretes a protease that acts on the competent cell to induce neural differentiation.

METHODS

Preparation

Embryos of *H. aurantium* were exclusively used except in the case of Fig. 2*A*, where *Halocynthia roretzi* embryos were also used. Fertilized eggs of *H. roretzi* were obtained by mixing eggs spawned from one animal with sperm from another. Fertilized eggs of *H. aurantium* were obtained by mixing

eggs dissected from one animal and sperm from another, as described previously (Okado & Takahashi, 1990*a*, *b*). The fertilized eggs were cultured in sea water at 9–10 °C until the 8- or 16-cell stage, and their chorionic membranes were stripped with fine tungsten needles. Each naked embryo was transferred into sea water containing 2 μg/ml cytochalasin B (Schroeder, 1978) to arrest cleavage. Blastomeres of dechorionated embryos at the 8-cell or 16-cell stage were separated mutually with a fine glass needle on a 0·4 % agarose-coated surface, as described previously (Okado & Takahashi, 1990*a*). The identification of each blastomere at the 8-cell or 16-cell stage was made according to the description by Conklin (1905), as described previously (Hirano *et al.* 1984). The blastomeres isolated with fine glass needles were transferred into Millipore (0·22 μm)-filtered sea water containing cytochalasin B (2 μg/ml, Aldrich), streptomycin (20 μg/ml) and penicillin (20 μg/ml) in 0·4 % agarose-coated microwells and cultured at 9–10 °C. The concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml about 2·5–3·5 h after isolation.

Determination of differentiation type by membrane excitability

When the control intact embryo developed and became a tadpole larva after about 60 h, the differentiated phenotypes of the cleavage-arrested blastomeres were examined electrophysiologically. The external solution consisted of (mM): NaCl, 400; $SrCl_2$, 100; KCl, 10; Pipes-Na (piperazine-*N*-*N*′-bis(2-ethanesulphonic acid)), 5 (pH = 7·0); and 0·1 % crystallized bovine serum albumin (Sigma). The blastomere was penetrated with a single microelectrode and the membrane potential held at −80 mV by applying constant current (Fig. 1). A depolarizing current pulse of 120 ms duration was applied to generate action potentials. Blastomeres that developed spikes (Fig. 1*A*; Okado & Takahashi, 1990*b*), composed of Na^+, Ca^{2+} and delayed K^+ channel currents, were regarded as having a neural phenotype. Blastomeres with long-lasting Sr^{2+} action potentials due to Ca^{2+} channels (Fig. 1*B*) or which were difficult to penetrate because of the presence of tunic membrane on the surface, which is an epidermal marker, were regarded as having an epidermal phenotype. The latter type of blastomere was found to express an epidermal antigen contained in tunic, while the former did not (Okado & Takahashi, 1990*a*, *b*). When no regenerative response was observed (Fig. 1*D*), blastomeres were regarded as being non-excitable, and were considered to have an incompletely developed epidermal phenotype or to have been injured during penetration. When evidence of an A-current was observed as a notch in the rising phase of a Ca^{2+} (Sr^{2+})-dependent regenerative response (Fig. 1*C*), the blastomere was regarded as being of the A-current type or type-II reported previously (Okado & Takahashi, 1990*a*). Only when the determination of type was obscure under current-clamp conditions, was voltage clamp applied by inserting another microelectrode as described previously (Okado & Takahashi, 1990*a*).

Treatment with proteases

As potential inducing agents for neural differentiation, various proteases were applied to the isolated and cleavage-arrested blastomere, $a_{4\cdot2}$, at the 32-cell stage of the intact embryo. Pronase was purchased from Kaken Chemical Co. Ltd (Tokyo, Japan) as 'Actinase E'. It was prepared as a 3 % solution in sea water, dialysed against sea water at 3 °C and stored at −70 °C. It was diluted with sea water just before use. Trypsins type I (10000 BAEE unit/mg, chymotrypsin < 4 BAEE), type II (1000–2000 BAEE unit/mg, chymotrypsin 1000–2000 ATEE unit/mg), type III (10000–13000 BAEE unit/mg, chymotrypsin < 4 BAEE), type XI (diphenylcarbamyl chloride treated, 7500–9000 BAEE unit/mg chymotrypsin < 0·1 BAEE), type XIII (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated, 10000–13000 BAEE unit/mg, chymotrypsin < 0·1 BAEE), chymotrypsins type II (40–60 unit/mg), type VII (*Nα*-*p*-tosyl-L-lysine chloromethyl ketone treated, 40–60 unit/mg), collagenase type I and ficin (2·25 % solution, 2·0 M NaCl and 0·03 M cistein) were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Subtilisin and proteinase K were obtained from Boehringer-Mannheim Biochemicals. Ficin was dialysed against sea water at 4 °C and diluted to 2·0 or 0·5 % before use. The others were dissolved in sea water before use.

Treatment with protease inhibitors

Streptomyces subtilisin inhibitor (SSI), was a gift from Professor Emeritus Ishii (Faculty of Pharmaceutical Sciences, Hokkaido University) and Professor Mitsui (Center for Physical Sciences, Nagaoka Institute of Technological Sciences). It was directly dissolved in sea water before use or a 0·2 % stock solution in sea water, which was stored at −20 °C, was diluted before use. Phenylmethyl-sulphonyl fluoride (PMSF), α1-antitrypsin, α2-macroglobulin and pepstatin, were purchased from Boehringer-Mannheim Biochemicals, antipain, chymostatin and pepstatin, E-64 from

Peptide Institute Inc. (Osaka, Japan) and aprotinin and diisopropyl fluorophosphate (DFP) from Sigma. Pepstatin was dissolved at 2% in methanol and stored at −20 °C. E-64 was dissolved at 2% in 50% ethanol and stored at −20 °C. DFP was dissolved at 1 M in absolute isopropanol and stored at 4 °C. PMSF was dissolved at 1 M in absolute dimethylsulphoxide (DMSO). Chymostatin was dissolved at 0·1 M in absolute DMSO and stored at −20 °C. Other inhibitors were dissolved directly in sea water.

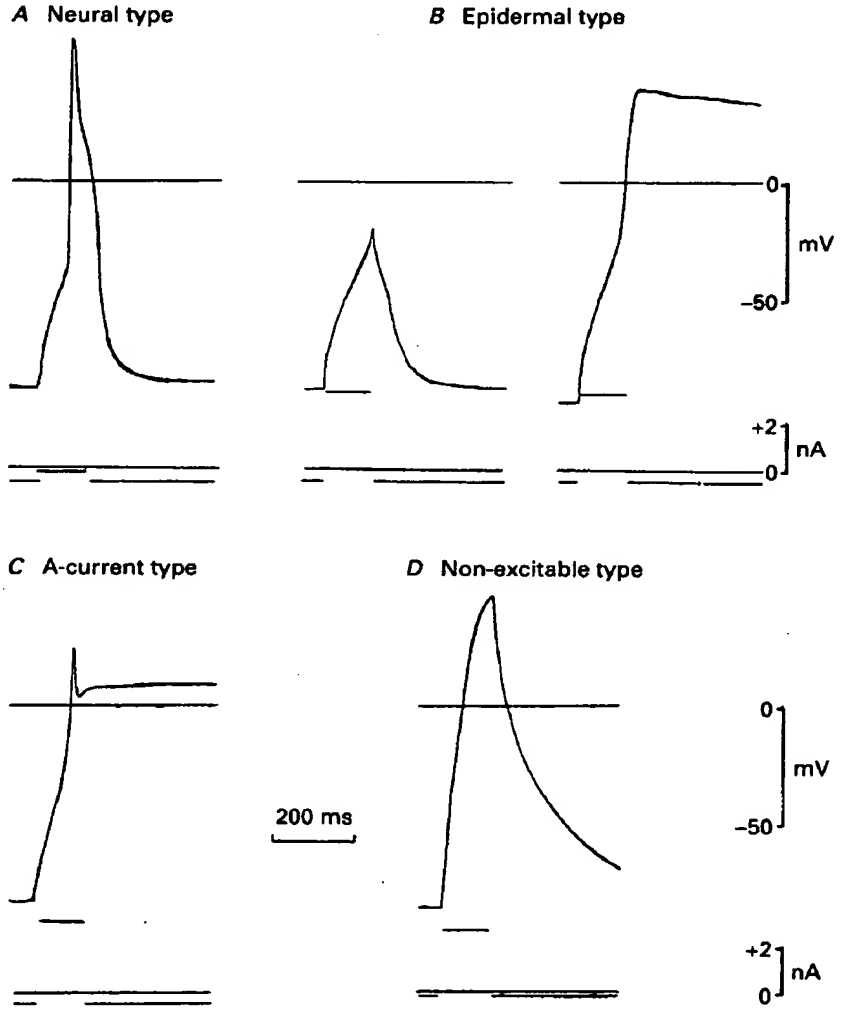


Fig. 1. Examples of the electrical responses observed in differentiated blastomeres which were isolated from 16-cell embryos, cleavage-arrested and cultured. *A*, neural type differentiation in an $a_{5\cdot4}$ blastomere induced by contact with $A_{4\cdot1}$. *B*, epidermal type differentiation observed in a $b_{5\cdot4}$ blastomere by contact with $A_{4\cdot1}$. *C*, A-current type differentiation in a $b_{5\cdot4}$ blastomere by contact with $A_{4\cdot1}$. *D*, non-excitable type observed in a $b_{5\cdot4}$ blastomere induced by subtilisin at 0·005% for 60 min. All records made under current clamp. Upper and lower traces are potential and current records respectively.

PMSF- and DFP-treated pronase solutions were made as follows. Stock solution of PMSF or DFP was added to 0·3% pronase in sea water to give a final concentration of 1–10 mM. The mixture was incubated at 25 °C for 1 h and dialysed against sea water at 4 °C.

Treatment with monensin, brefeldin A or bafilomycin A1

Monensin (sodium salt, Sigma) was dissolved at 20 mM in ethanol and stored at −20 °C. For use it was diluted in sea water to 1·0 or 0·1 μM. Brefeldin A (Sandoz Company, Basel, Switzerland) was stored as a 10 mg/ml stock solution at −20 °C in absolute methanol. Bafilomycin A1 was kindly given by Dr Y. Moriyama (Osaka University) as a 1 mM solution in absolute ethanol.

RESULTS

Neural inducing ability of proteases

As reported previously (Okado & Takahashi, 1990*b*), the cleavage-arrested blastomere, $a_{4\cdot2}$, isolated from ascidian embryos was induced to differentiate into a neural-type cell by treatment with pronase. The dose–response curve in Fig. 2*A* shows that the $a_{4\cdot2}$ blastomere from *H. aurantium* was induced more readily than that from *H. roretzi*. For example, 0·02% pronase treatment for 15 min at the 32-cell stage induced 90% of the $a_{4\cdot2}$ blastomeres of *H. aurantium*, but only 10% of *H. roretzi*. Subsequent experiments were performed using the more sensitive blastomeres, that is, those of *H. aurantium*.

Because pronase is a mixture of proteolytic enzymes produced by *Streptomyces griseus*, we tried to separate the individual proteases by chromatography on a CM-cellulose column according to Narahashi (1970) and to identify neural inducing proteases. When the neural inducing activity of each fraction was examined, it was found that neural inducing ability of proteases did not always reflect common protease activity measured with the casein–275 nm method (Narahashi, 1970). There were two vague peaks of inducing activity, corresponding to the neutral protease and alkali protease peaks reported previously by Narahashi (1970).

Since it is known that pronase consists for the most part of serine proteases, we examined whether the inducing ability of pronase was inhibited by treatment of serine protease inhibitors, such as DFP (not shown) or PMSF (Fig. 2*B*). We found that these inhibitors could reduce the inducing activity of pronase, but did not block it completely. For example, 10 mM PMSF inhibited the inducing activity of 0·2% pronase by 50%, although the percentage inhibition was greater at lower concentrations of pronase (Fig. 2*B*). This result suggested that not only serine proteases but also non-serine proteases in pronase had inducing ability.

Because it is difficult to interpret the effect of a mixture of proteases, as found in pronase, we examined the inducing activity of various purified proteases (Fig. 2*C*). Since we applied enzymes in the concentration range from 0·005 to 2% and for durations of 15 or 60 min, the dose of enzymes was represented by concentration (%) multiplied by duration (min) as a unit in the graph (Fig. 2*C*). One serine protease, subtilisin (shown as filled triangles), had similar inducing ability to pronase (shown as a dashed line). The other serine proteases, trypsin and chymotrypsin, had less activity. The trypsins without contamination of chymotrypsin (types XI and XIII) had less inducing activity than crude trypsin (type II), although the former had more trypsin activity (BAEE activity) than the latter, indicating that the specific proteolytic activity of trypsin has poor neural inducing activity. The crude chymotrypsin (type II) and that without trypsin (type VII) also had low activity. Even a mixture of purified trypsin (type XIII) and chymotrypsin (type VII) showed

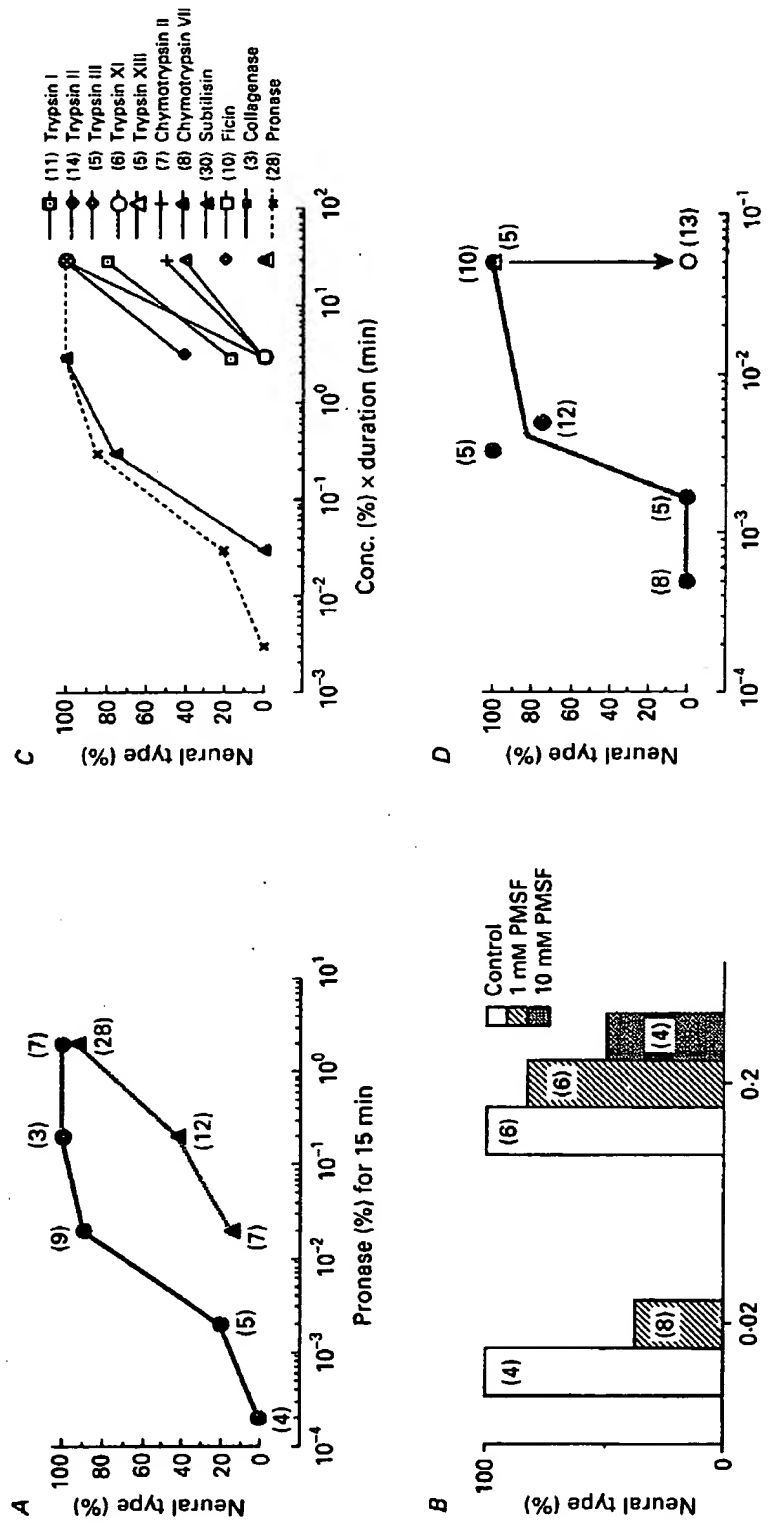


Fig. 2. *A*, dose–reponse curves of neural inducing activity of pronase (Actinase E, Kaken). Cleavage-arrested blastomeres, $a_{4\cdot2}$, isolated from 8-cell embryos of *Halocynthia roretzi* (▲) and *Halocynthia aurantium* (●) were treated with pronase at various concentrations for 15 min at the equivalent of the 32-cell stage of the control embryo, that is, 2·5 h after the 8-cell stage at 10 °C. *B*, effect of serine protease inhibitor (PMSF) upon the neural inducing activity of pronase. PMSF-treated pronase solution was prepared as described in the Methods. *C*, neural inducing activity of various proteases. The data for the dashed line (pronase) is derived from *A* ( ). The abscissa represents dose of protease calculated as percentage concentration times duration of application (min). Figures in parentheses on right side are sample numbers for experiments of respective enzymes. *D*, dose–response curve of neural inducing activity of subtilisin (●). ○, 0·05 % SSI blocked neural induction produced by 0·05 % subtilisin. △, 0·005 % SSI had no effect on induction. Cleavage-arrested blastomeres, $a_{4\cdot2}$, isolated from 8-cell embryos of *Halocynthia aurantium* were treated with subtilisin at various concentrations for 60 min at the 32-cell stage; data are combined from embryos of four batches. In *A*, *B* and *D* the figures in parentheses are the numbers of samples used to calculate the respective percentage frequencies for plotting or drawing columns.

much less effect, indicating that neither trypsin nor chymotrypsin are candidates for the potent inducing protease. The thiol protease, ficin, could induce neural differentiation of blastomeres only when used at a higher concentration (0·5%) for 60 min, which was 100-fold higher than the amount of purified subtilisin (0·005% for 60 min) required for neural induction. The fact that proteases other than serine proteases had inducing ability may be consistent with the observed partial inhibition of neural inducing activity of pronase by the serine protease inhibitors, DFP, or PMSF (Fig. 2*B*). The metal protease, collagenase, had no inducing activity even at 0·5% for 60 min. Another dose–response curve of subtilisin was separately obtained as shown in Fig. 2*D*; in this experiment subtilisin was applied for 60 min at the 32-cell stage of the control embryo. The threshold contration for Na^+ spike induction was about 0·003% or 1·15 μM. The effect of subtilisin (0·05% or 19 μM) was blocked completely by the same concentration of *Streptomyces* subtilisin inhibitor (SSI, 0·05% or 44 μM as monomer), while SSI at the one-tenth of this concentration (0·005% or 4·4 μM) could not suppress the effect at all (Fig. 2*D*, open circle, open triangle and arrow; Tonomura, Sato, Miwa & Komiyama, 1985). The stoichiometry of the inhibition is known to be two molecules of subtilisin bound to the dimeric SSI (Tonomura *et al.* 1985). Therefore the neural inducing activity is due to the proteolytic activity of subtilisin itself. SSI could not inhibit the inducing ability of pronase at the same concentration. Hereafter, all experiments were done with subtilisin as the inducing protease.

Time sequence of changes in neural competence of $a_{4\text{-}2}$

In a previous study (Okado & Takahashi, 1990*b*), we showed that the receptiveness of the $a_{4\text{-}2}$ to pronase as well as competence to induction by contact was lost during the gastrula stage. We found, however, that sensitivity to pronase (2%, 15 min) existed as early as the 8-cell stage, while competence to induction by contact appeared only after the 32-cell stage. Thus, this discrepancy must be explained before we can determine the mechanisms underlying the two modes of induction. In the present study, by using various concentrations of subtilisin and SSI (Tonomura *et al.* 1985), the precise time sequence of changes in neural competence was examined. In the case of subtilisin at 0·05% for 60 min, the blastomere $a_{4\text{-}2}$ was induced at the 8-cell stage to differentiate into a neural-type cell (filled circles) (Fig. 3*A*). This result with subtilisin (0·05%, 60 min) is consistent with the previous result with pronase (2%, 15 min). In Fig. 3 the dashed lines represent neural competence of $a_{4\text{-}2}$ with induction by contact, which was reported previously (Okado & Takahashi, 1990*b*). It was possible that the discrepancy between the time courses of neural competence by protease and by cell contact might result from an overdose of subtilisin which could cause a saturating effect even in a slightly competent phase. To test this possibility, the concentration of subtilisin was reduced to 0·005%. In this situation, at the 8-cell stage the $a_{4\text{-}2}$ did not differentiate neurally, but epidermally (Fig. 3*B*). Therefore, the discrepancy comes at least partially from the overdose of subtilisin. Another possibility is that some protease remained after washout with sea water and that this remaining protease showed residual activity subsequent to the time of treatment. In order to test this possibility, the blastomere was washed, after subtilisin treatment, with sea water containing SSI for 60 min (Fig. 3*C*). This

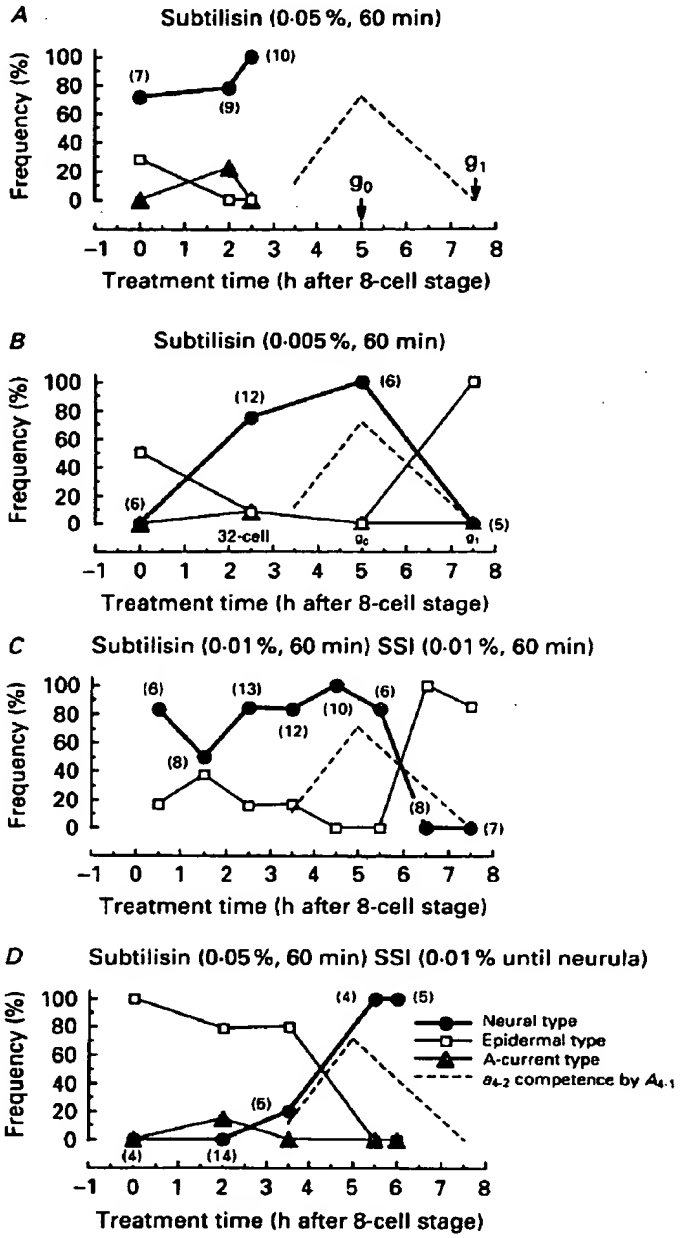


Fig. 3. Time sequence of changes in neural competence of $a_{4\cdot2}$ blastomeres isolated from 8-cell embryos of *H. aurantium* induced by treatment with subtilisin. The dashed lines represent the time sequence of neural competence of $a_{4\cdot2}$ induced by contact with $A_{4\cdot1}$, which was reported previously (g_0 and g_1 are early and middle substages within the gastrula stage; Okado & Takahashi, 1990*b*). ●, neural development. ▲, A-current type (previously called type-II; Okado & Takahashi, 1990*b*) development. □, epidermal type development. Temperature was 9–10 °C in *A*, 10 °C in *B* and *C*, and 9 °C in *D*. *A*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·05 % subtilisin for 60 min at various stages. *B*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·005 % subtilisin for 60 min at various stages. *C*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·01 % subtilisin for 60 min at various stages, and then they were immersed in sea water containing 0·01 % SSI for 60 min to wash out the remaining subtilisin. *D*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·05 % subtilisin for 60 min at

procedure had little effect. In order to suppress the remaining protease activity completely after washing, the blastomere was kept in sea water containing SSI until about the neurula stage (Fig. 3*D*). This experiment showed that the rising phase of neural competence induced by subtilisin agreed with that induced by contact with $A_{4\cdot1}$. These results indicate that treatment with subtilisin mimicked contact with $A_{4\cdot1}$ well with regard to the time sequence of neural competence, both in the rising and fading phases. The applied subtilisin was difficult to eliminate by washing in sea water alone or in sea water containing SSI for a limited time, such as 60 min.

Regional specificity of ectodermal blastomeres

In addition to the anterior ectodermal blastomere, $a_{4\cdot2}$, the posterior $b_{4\cdot2}$, isolated from the 8-cell embryo, was also neurally induced by contact with the anterior-vegetal blastomere $A_{4\cdot1}$, though less frequently, as described previously (Okado & Takahashi, 1990*b*). Thus, there was clear regional specificity of ectodermal blastomeres with respect to neural competence. It is important to determine whether this regional difference in competence was also observed by induction with protease. For this purpose, four ectodermal blastomeres isolated from 16-cell embryos, anterior $a_{5\cdot3}$, $a_{5\cdot4}$, posterior $b_{5\cdot3}$ and $b_{5\cdot4}$, located in order on the antero-posterior axis, were studied to examine more precisely the regional differences in neural competence along this axis and the results obtained from two modes of induction were compared. The isolated blastomeres from 16-cell embryos were cultured and placed in contact with the same inducing blastomere $A_{4\cdot1}$ (isolated from the 8-cell stage) at the 64-cell stage of the control embryo. Two main differences were observed among the blastomeres. First, $a_{5\cdot3}$ and $a_{5\cdot4}$ were induced to differentiate neurally with high frequency ($> 80\%$), while $b_{5\cdot3}$ and $b_{5\cdot4}$ were induced with only low frequency ($< 40\%$) (Fig. 4). Second, A-current-type spikes developed characteristically in $b_{5\cdot3}$ and $b_{5\cdot4}$. These results indicated that the neural competence of ectoderm is already specified at the 16-cell stage. In fact, a previous study with pronase (Okado & Takahashi, 1990*b*) indicated that as early as the 8-cell stage neural competence of the isolated $a_{4\cdot2}$, which is the parent cell of $a_{5\cdot3}$ and $a_{5\cdot4}$, is higher than that of the isolated $b_{4\cdot2}$, which is the parent cell of $b_{5\cdot3}$ and $b_{5\cdot4}$. In $b_{4\cdot2}$ the probability of Na^+ spike induction was relatively low, while the A-current-type spikes were observed relatively frequently (Okado & Takahashi, 1990*b*). Since A-current-type spikes were generally observed in $A_{4\cdot1}$ blastomeres from the 8-cell embryo (Okado & Takahashi, 1990*a*) and $b_{4\cdot2}$ and $A_{4\cdot1}$ both include presumptive spinal cord, the caudal portion of neural tissue of the tadpole larva (Nishida, 1987), it is inferred that A-current type spikes might be a cell membrane marker of spinal cord and/or caudal neural tissue.

various stages, and then they were immersed in sea water containing 0·1 % SSI until the neurula stage of intact embryos to inhibit activity of subtilisin completely. Cytochalasin B was reduced from 2 to 0·2 μg/ml at the neurula stage. Abscissa, hours after the isolation of blastomeres at 8-cell stage. Thus, 2·5 h corresponds to 32-cell stage and plus 5 h corresponds to the transition from the 64-cell to the 110-cell stage of the control embryo. Data from embryos of six batches were combined. Ordinate, percentage frequencies of respective differentiation types indicated by the induction of Na^+ spikes, Ca^{2+} (Sr^{2+})-dependent epidermal type action potential or A-current type spikes. The figures in parentheses are sample numbers used for calculation of percentage frequencies at respective developmental times.

Actually the A-current type spikes were also observed in $a_{4\cdot2}$, although exceptionally, when the enzyme was applied at earlier stages such as the 8-cell or 16-cell stage, as shown in Fig. 3*A*, *B* and *D* (filled triangles; Okado & Takahashi, 1990*a*).

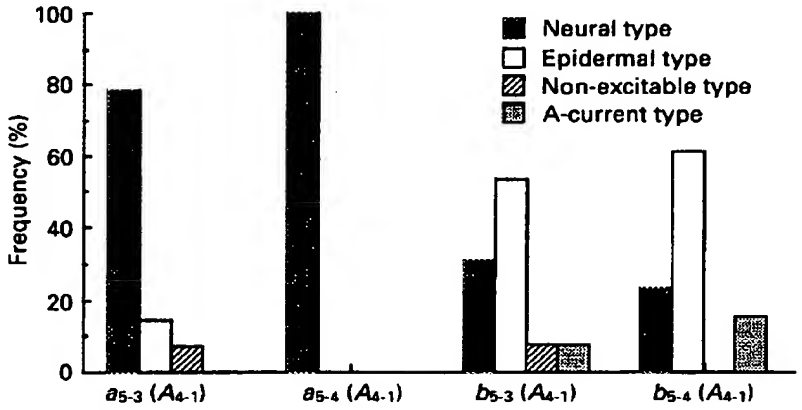


Fig. 4. Neural competence of isolated and cleavage-arrested ectodermal blastomeres of 16-cell embryos of *H. aurantium* induced by contact with the $A_{4\cdot1}$ blastomere from the 8-cell embryo. The ectodermal blastomeres, $a_{5\cdot3}$ (14 cells), $a_{5\cdot4}$ (10), $b_{5\cdot3}$ (13) and $b_{5\cdot4}$ (13) isolated from 16-cell embryos were placed in contact with $A_{4\cdot1}$ blastomeres isolated from 8-cell embryos at about the 64-cell stage of the control embryo, i.e. 2 h after the 16-cell stage. Data from embryos of three batches were combined.

Since we did not know the real inducing activity of $A_{4\cdot1}$ in cell contact, it was necessary to analyse competence with subtilisin as an inducer at various levels of enzyme concentration (Fig. 5). The results were summarized as follows. First, regional differences in neural competence were revealed at subtilisin concentrations greater than 0·003 %, which is the threshold concentration for Na^+ spike development for all of the blastomeres except the $b_{5\cdot4}$. Second, the order of neural competence of the four blastomeres studied was $a_{5\cdot3} > a_{5\cdot4} > b_{5\cdot3} >= b_{5\cdot4}$. In blastomeres $b_{5\cdot3}$ and $b_{5\cdot4}$ the frequency of neural development was saturated at a level of less than 50 % even when subtilisin was applied at concentration greater than 0·01 %. Third, in $b_{5\cdot3}$ and $b_{5\cdot4}$ A-current-type spikes developed following treatment with subtilisin (Fig. 5, $b_{5\cdot3}$, $b_{5\cdot4}$) as in the case of induction by contact with $A_{4\cdot1}$, but such spikes did not appear in $a_{5\cdot3}$ and $a_{5\cdot4}$. In addition to the epidermal type definitely identified by the presence of Ca^{2+}(Sr^{2+}) action potentials and/or tunic, the non-excitable type was frequently observed in the $b_{5\cdot3}$ and $b_{5\cdot4}$ following treatment with subtilisin at more than 0·003 %, but we considered that this type may represent an incomplete form of epidermal development. A type of differentiation with only delayed outward current was observed in the case of $b_{5\cdot3}$ and $b_{5\cdot4}$ treated with 0·2 % subtilisin. We considered that a part of this type is an incomplete form of A-current-type spikes because the outward current was frequently associated with fast inactivation. Some injurious effects of the high dose treatment with subtilisin may be one of the reasons why incomplete forms appeared in significant numbers. However, it should be noted that Na^+ spikes induced in $a_{5\cdot3}$ and $a_{5\cdot4}$ with 0·2 % subtilisin were well developed, comprising large Na^+ and delayed K^+ currents as reported previously (Okado & Takahashi, 1990*b*).

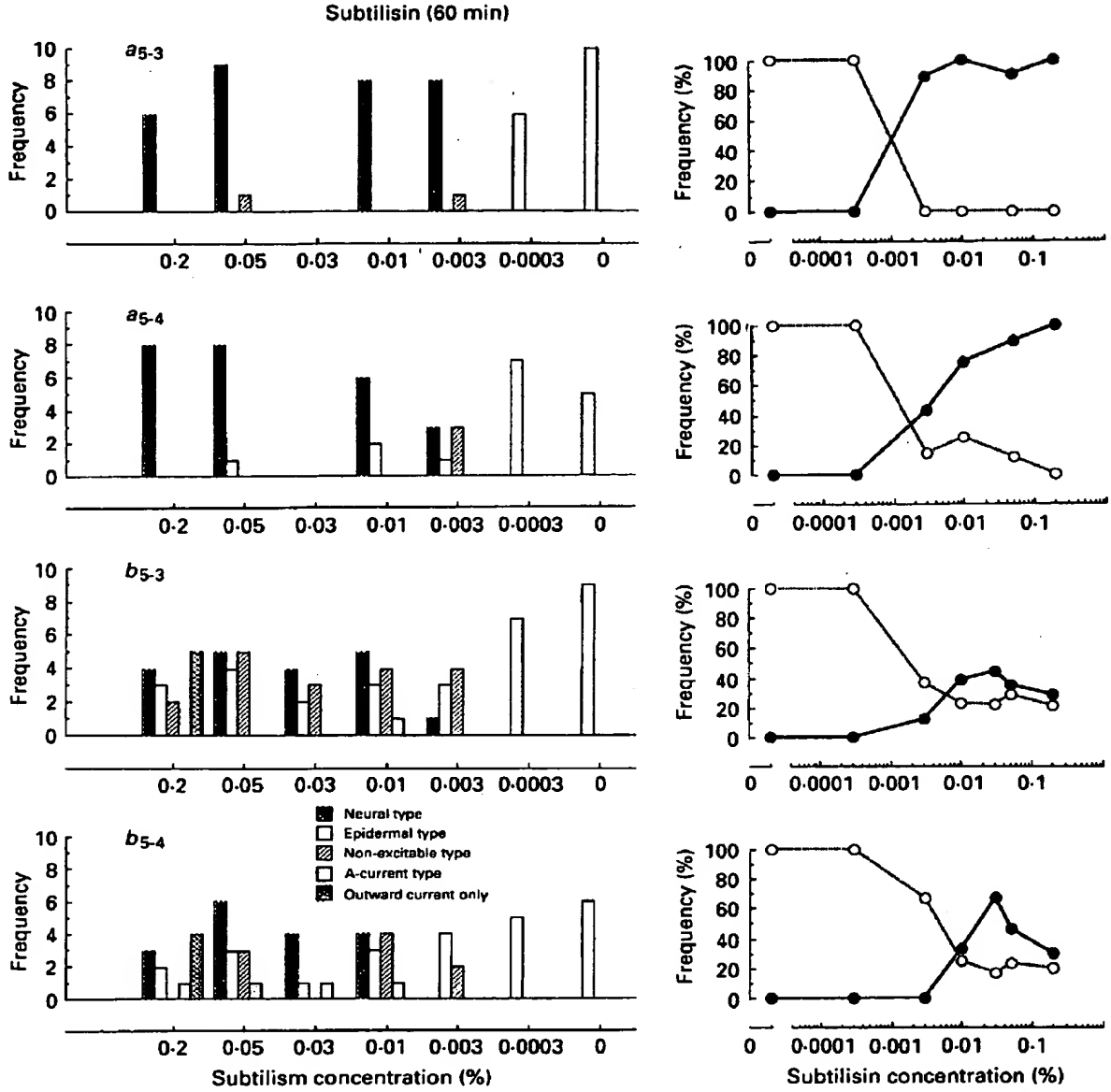


Fig. 5. Neural competence of cleavage-arrested ectodermal blastomeres isolated from 16-cell embryos of *H. aurantium* and induced by subtilisin. The ectodermal blastomeres, $a_{5\cdot3}$ (49 cells), $a_{5\cdot4}$ (44), $b_{5\cdot3}$ (74) and $b_{5\cdot4}$ (58) isolated from 16-cell embryos were cleavage-arrested and treated with subtilisin at various concentrations from 0 to 0·2% for 60 min, at about the 64-cell stage of control embryos, i.e. 2 h after the 16-cell stage. Data from embryos of seven batches were combined. On the left side, are frequency histograms of the various differentiated phenotypes induced at various subtilisin concentrations. In the graphs on the right side, percentage frequencies of neural-type (●) and epidermal-type development (○) shown in the histograms were plotted against the dose of subtilisin on a semilogarithmic scale.

The percentage frequencies of defined neural- and epidermal-type development shown in Figs 4 and 5 are plotted according to the location of the blastomeres along the antero-posterior axis in Fig. 6*A* and *B* both for induction by subtilisin and by contact. Here, the percentage frequencies were considered to represent potencies of

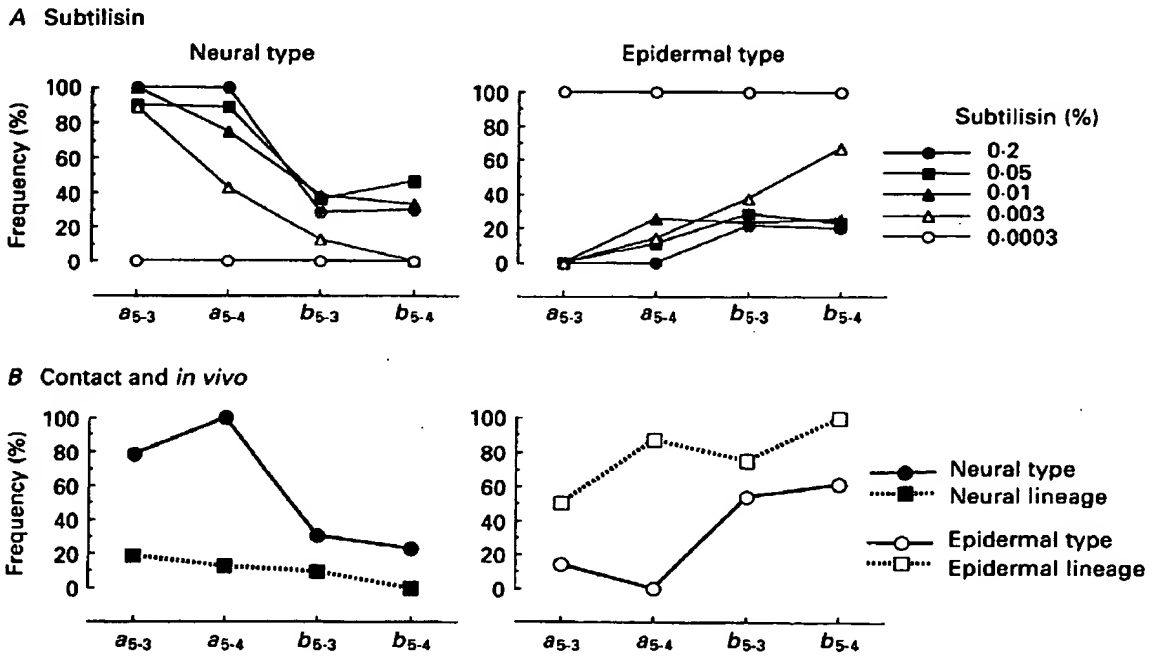


Fig. 6. Regional differences in neural competence in ectodermal blastomeres isolated from 16-cell embryos. Percentage frequencies of neural and epidermal phenotypes are plotted according to the order of blastomeres along anterior–posterior axis. *A*, percentage frequency of neural and epidermal development induced by subtilisin was plotted against the location of blastomeres, calculated from the data in Fig. 5. *B*, percentage frequency of neural- and epidermal-type development induced by contact with $A_{4\text{-}1}$ was plotted similarly (calculated from the data shown in Fig. 4). The dotted lines with filled and open squares represent percentage ratios of cell numbers of presumptive neural and epidermal lineages respectively at the 110-cell stage derived from each ectodermal blastomere of the control 16-cell embryo for comparison with the regional differences in neural competence. The ratios were calculated tentatively, based on the differentiation type of derivatives from respective blastomeres according to the data reported by Nishida (1987). Brain, brainstem and pigment cells were regarded as neural tissues in the calculation. It is known that at the 110-cell stage most of the blastomeres are restricted to express a particular tissue (Nishida, 1987). However, where two or three kinds of tissue are expressed after development in the derivatives from a blastomere at the 110-cell stage, the number for the respective lineage was counted as a half or a third.

neural or epidermal competence of the respective blastomeres. Dotted lines in Fig. 6*B* illustrate the inferred ratio of presumptive neural and epidermal lineages at the 110-cell stage, which are derived from respective blastomeres of the 16-cell embryos according to cell lineage studies in *H. roretzi* by Nishida (1987) obtained with HRP (horseradish peroxidase) as a tracer. The number of cells neural lineage derived from ectodermal blastomeres decreases in the order $a_{5\text{-}3}$, $a_{5\text{-}4}$, $b_{5\text{-}3}$, $b_{5\text{-}4}$, assuming that brain,

brainstem and pigment cell are regarded as neural tissues (Nishida, 1987). The results shown in Fig. 6 led us to the following two conclusions. First, the regional difference of neural competence by subtilisin was similar to that by contact with $A_{4\cdot1}$, and, thus, the effect of subtilisin closely mimicked that of contact with $A_{4\cdot1}$, which

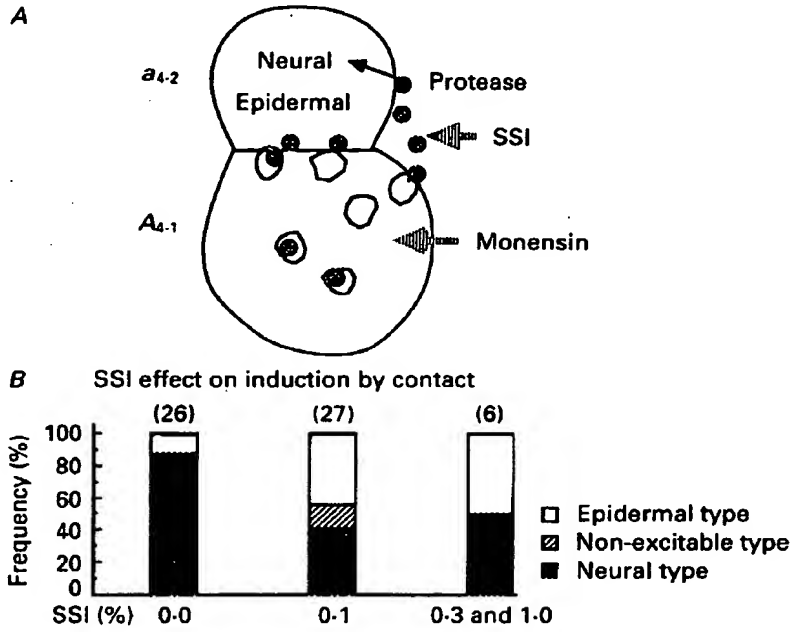


Fig. 7. *A*, hypothesis for neural induction by secreted proteases and schematic illustration for effects of externally applied SSI or monensin. *B*, effect of SSI on neural differentiation of $a_{4\cdot2}$ blastomeres induced by contact with $A_{4\cdot1}$. The figures in parentheses are sample numbers for respective concentrations of SSI. The samples for 0·3 and 1·0% were combined because there were only three samples for each concentration.

was regarded as the natural inducing stimulus *in vivo*. Second, the regional specificity for neural differentiation in normal development, as indicated by the difference in neural lineage populations derived from respective blastomeres (Fig. 6*B*), was largely paralleled by the regional difference of neural competence examined by either the foreign inducer, subtilisin, (Fig. 6*A*) or the natural inducing stimulus, contact with $A_{4\cdot1}$ (Fig. 6*B*). This result suggested that neural competence was predetermined ahead of induction, probably by cytoplasmic segregation among blastomeres as early as at the 16-cell stage; that is likely also to be the case for neural development *in vivo*. However, Nishida (1987) showed in his cell lineage studies that $b_{5\cdot4}$ generates no neural tissue *in vivo*, while the present study clearly showed that $b_{5\cdot4}$ was as neurally competent as $b_{5\cdot3}$. This point will be discussed later.

Effect of various protease inhibitors

The above results led us to hypothesize that the inducing substance is a protease that it is secreted from $A_{4\cdot1}$, and that it induces the $a_{4\cdot2}$ cell to differentiate into neural cell type (Fig. 7*A*). To examine whether proteases really are involved in the

neural induction of $a_{4\cdot2}$ by $A_{4\cdot1}$, various protease inhibitors were added to sea water in microwells, each of which contained an $a_{4\cdot2}$ and an $A_{4\cdot1}$ blastomere. The results obtained are illustrated in Table 1. They indicate that one of the specific serine protease inhibitors, SSI (Tonomura *et al.* 1985), and a wide-spectrum inhbiitor, α2-macroglobulin (Harpel, 1976), significantly inhibited neural induction. At 0·1 % SSI

TABLE 1. Effect of various protease inhibitors on neural induction by contact

Protease inhibitor	Mol. wt.	Concentration (% (mM))	Neural type (frequency (%))	Epidermal type (frequency (%))
Control			23/26 (88)	3/26 (12)
Serine protease inhibitors				
Aprotinin	6511	0·1 (0·15)	2/2 (100)	0/2 (0)
α1-antitrypsin	54000	0·4 (0·07)	3/4 (75)	0/ 4 (0)*
SSI	23000 as dimer	1·0 (0·440)	1/3 (33)	2/3 (67)
		0·3 (0·132)	2/3 (67)	1/3 (33)
		0·1 (0·044)	11/27 (41)	12/27 (44)*
DFP		(10)	5/5 (100)	0/5 (0)
		(1·0)	3/3 (100)	0/3 (0)
Chymostatin	*ca.* 600	0·03 (0·5)	5/5 (100)	0/5 (0)
		0·006 (0·1)	1/2 (50)	0/2 (0)*
Serine–thiol protease inhibitors				
Antipain	604	0·02 (0·3)	4/4 (100)	0/4 (0)
Leupeptin	460	0·5 (10)	2/2 (100)	0/2 (0)
		0·05 (1·0)	8/10 (80)	0/10 (0)*
Thiol protease inhibitor				
E-64	357	0·02 (0·5)	2/2 (100)	0/2 (0)
Aspartyl protease inhibitor				
Pepstatin	685	0·02 (0·3)	4/4 (100)	0/4 (0)
Wide-spectrum inhibitor				
α2-macroglobulin	*ca.* 800000 as tetramer	1·0 (0·0125)	1/7 (14)	2/7 (29)*
		0·3 (0·00375)	2/3 (67)	1/3 (33)
		0·1 (0·00125)	2/4 (50)	2/4 (50)

The $a_{4\cdot2}$ and $A_{4\cdot1}$ blastomeres were isolated from 8-cell embryos of *H. aurantium*, cleavage-arrested and cultured separately with 2 μg/cytochalasin B. Two hours after isolation the $A_{4\cdot1}$ cells were immersed in sea water containing the protease inhibitor. Two and half hours after isolation $a_{4\cdot2}$ cells were put in contact with the treated $A_{4\cdot1}$ cell, kept in sea water containing various protease inhibitors and cultured. At the neurula stage of the control embryo the $a_{4\cdot2}$ cell in contact with the $A_{4\cdot1}$ cell was transferred into protease inhibitor-free sea water. The concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml at the time of contact, i.e. 2·5 h after isolation. Reduction of cytochalasin B facilitates the contact (Okado & Takahashi, 1990*b*). Asterisks in the 'epidermal type' column indicate that blastomeres other than neural or epidermal type in respective experiments were of the non-excitable type which was likely to be an incomplete form of the epidermal phenotype.

inhibited neural induction in 50 % of cases, but even higher concentrations of SSI, 0·3 or 1 %, did not increase this level of inhibition (Fig. 7*B*; Table 1). α2-Macroglobulin at high concentration (1 %) inhibited neural development significantly, but definite epidermal type differentiation was relatively rare and a high percentage (57 %) of the blastomeres became non-excitable (Table 1). The other serine protease inhibitors,

thiol protease inhibitors and aspartyl protease inhibitors had no effect (Table 1). Incompleteness of inhibition with SSI could be explained in the following two ways: first, other proteases which were insensitive to the above inhibitors also contribute to neural induction; second, SSI may not have easy access to the intercellular space between the contacting membranes of $a_{4\cdot2}$ and $A_{4\cdot1}$ during the induction phase (Fig. 7*A*).

Effect of substances affecting secretion; monensin, brefeldin A and bafilomycin A1

Monensin, a carboxylic Na^+–H^+ ionophore isolated from *Streptomyces cinnamonensis*, is known to interfere with the intracellular pathway through the Golgi apparatus that is responsible for processing secretory proteins or assembling integral proteins into the membrane. It is also known that monensin causes a rapid swelling of the Golgi apparatus (Tartakoff, 1983). To test whether a secretory process is involved in neural induction, the effects of monensin on neural induction were examined. The $a_{4\cdot2}$ and $A_{4\cdot1}$ blastomeres isolated at the 8-cell stage were cultured separately and placed in contact with each other for 6 h from the 32-cell stage to the late gastrula stage, during which 0·1 μM monensin was applied (Fig. 8*A*). In all six cases examined monensin inhibited neural induction in $a_{4\cdot2}$ blastomeres and directed differentiation towards an epidermal phenotype. In five control experiments $a_{4\cdot2}$ blastomeres from the same batch were induced to differentiate neurally in all cases. To exclude the possibility that monensin interferes with neural development itself after induction, we showed that $a_{4\cdot2}$ blastomeres treated with subtilisin (0·05% for 60 min) developed a neural phenotype even when kept in sea water containing 0·1 μM monensin for 6 h after the treatment ($n = 6$, Fig. 8*B*). To determine whether monensin affected $a_{4\cdot2}$ or $A_{4\cdot1}$, either blastomere was kept in sea water containing monensin (1 μM for 60 min) and washed just before contact (Fig. 8*C* and *D*). Only when the inducing blastomere, $A_{4\cdot1}$, was treated with monensin was neural induction significantly suppressed, suggesting that some secretory process in the $A_{4\cdot1}$ was necessary for neural induction. Although monensin affected inductive activity of $A_{4\cdot1}$ it did not significantly alter the frequency of occurrence of the various phenotypes of $A_{4\cdot1}$ after differentiation, the blastomere showing A-current type spikes or epidermal type action potentials or becoming non-excitable, similar to those described previously (Okado & Takahashi, 1990*a*, *b*).

We also examined the effects of other secretion blocking agents: brefeldin A (Misumi, Misumi, Miki, Takatsuki, Tamura & Ikehara, 1986), which provokes dynamic resorption of most of the membranes of the Golgi apparatus into the endoplasmic reticulum and results in blocking transport of secretory proteins to post-Golgi compartments (Misumi *et al.* 1986; Lippincott-Schwartz, Yuan, Bonifacino & Klausner, 1989), and bafilomycin A1 (Umata, Moriyama, Futai & Medkada, 1990), which is a specific inhibitor of vacuolar-type H^+-ATPases and possibly blocks transport processes in the Golgi apparatus. Both brefeldin A (10 μg/ml) and bafilomycin A1 (1 μM) inhibited the neural inducing ability of $A_{4\cdot1}$. When $A_{4\cdot1}$ blastomeres were pre-treated with brefeldin A for 60 min and washed before being placed in contact with $a_{4\cdot2}$ blastomeres, no $a_{4\cdot2}$ blastomeres differentiated neurally; they all developed an epidermal phenotype ($n = 5$). Treatment of $A_{4\cdot1}$ blastomeres with a lower concentration (1 μg/ml) of brefeldin A showed a much less

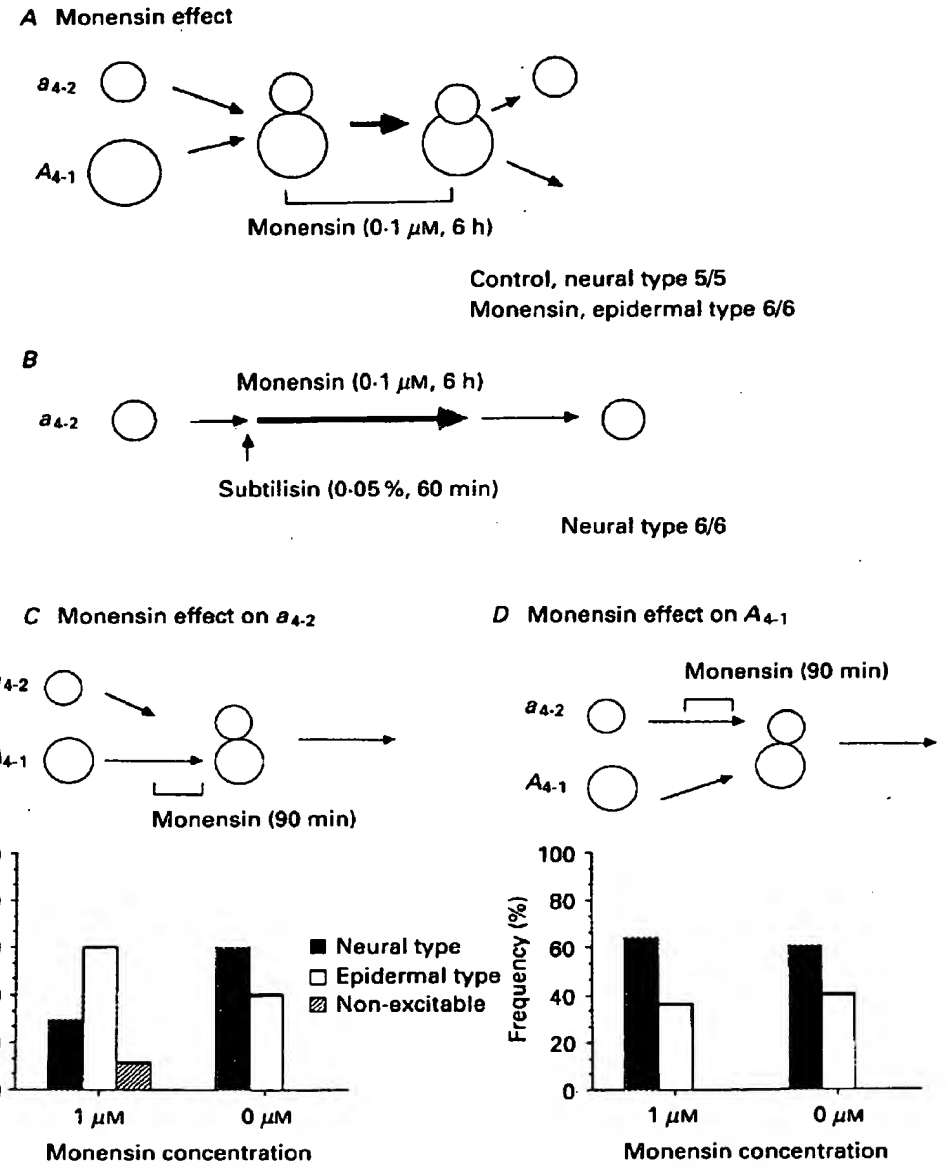


Fig. 8. Effect of monensin on $a_{4\cdot2}$ and $A_{4\cdot1}$ of *H. aurantium* embryo during neural induction. *A*, the cleavage-arrested and isolated $a_{4\cdot2}$ blastomere was contacted by the $A_{4\cdot1}$ blastomere at the 32-cell stage of control embryos in sea water containing 0·1 μM monensin for 6 h. They were then washed, separated and cultured without monensin. All of six cases failed to differentiate neurally, but differentiated epidermally. Control experiments without monensin were similarly performed with blastomeres from the same batch of embryos. All of five cases differentiated neurally. *B*, the $a_{4\cdot2}$ was treated with 0·05% subtilisin for 60 min at the 32-cell stage of control embryos, and transferred into sea water containing 0·1 μM monensin for 6 h. In all of six cases $a_{4\cdot2}$ differentiated neurally. *C*, the $A_{4\cdot1}$ was immersed in sea water containing 1 μM monensin for 90 min about 1 h after the 8-cell stage before contacting with $a_{4\cdot2}$. The lower part shows a histogram of percentage frequencies of differentiation type; $n = 17$ for the experiment and $n = 15$ for the control (0 μM). *D*, the $a_{4\cdot2}$ was treated before contact. The procedure was similar to that in *C*; $n = 11$ for the experiment and $n = 15$ for the control (0 μM). The data from three batches of embryos were combined for Fig. 8*C* and *D*. In all cases, the

significant effect, with two out of four contacted $a_{4\cdot2}$ blastomeres adopting a neural phenotype. To rule out direct epidermalizing effects on $a_{4\cdot2}$ by an brefeldin A that might remain on the $A_{4\cdot1}$ after washing, four $a_{4\cdot2}$ blastomeres neurally induced by subtilisin (0·05% for 60 min) were treated directly by applying brefeldin A at a concentration of 10 μg/ml for 10 h. Three (two with Na^+ spikes and one with delayed K^+ current only) out of four became neural in type and one became non-excitable. This shows that direct application of brefeldin A slightly suppressed neural development. However, it seems unlikely that the amount of brefeldin A that might remain on $A_{4\cdot1}$ blastomeres would have direct effects on $a_{4\cdot2}$. The suppressing effect of bafilomycin A1 (1 μM) on $A_{4\cdot1}$ was relatively weak. Three out of eleven $a_{4\cdot2}$ blastomeres, which were in contact with $A_{4\cdot1}$ blastomeres treated by bafilomycin A1 (1 μM for 60 min) before contact, differentiated into epidermal type, three differentiated neurally and the remaining five showed small regenerative responses possibly due to Ca^{2+} (Sr^{2+}) currents. When four $a_{4\cdot2}$ blastomeres induced by subtilisin (0·05% for 60 min) were treated with the same concentration of bafilomycin A1, they all differentiated neurally, indicating no direct effect of the drug on $a_{4\cdot2}$. All the above results suggest that neural induction requires some secretory processes or transport processes through the Golgi apparatus in the inducer blastomere, $A_{4\cdot1}$.

DISCUSSION

Protease as a neural inducer

In the present experiments we found that subtilisin, a serine protease, had potent neural inducing activity on the ascidian presumptive neural blastomere, $a_{4\cdot2}$, and mimicked the *in vivo* inducing activity of $A_{4\cdot1}$ blastomeres by showing both a similar time sequence of changes in neural competence of $a_{4\cdot2}$ blastomeres, and the same regional difference in neural competence among ectodermal blastomeres of 8- or 16-cell embryos. Further, it was found that natural induction by contact with $A_{4\cdot1}$ could be partially suppressed by the subtilisin inhibitor, SSI.

In 1979, it was reported that trypsin, another serine protease, can induce some neural structures, namely pigment spots and a tissue-specific enzyme, brain pigment cell tyrosinase, in animal quartets isolated from 8-cell embryos of *Ascidia maldia* and *Phallusia mamilata*, and cultured independently of the natural inducer in the anterior-vegetal quartet (Ortolani, Patricolo & Mansueto, 1979). Since, in our case, the inducing ability of subtilisin was greater than that of trypsin, it is safe to conclude that some proteases, possibly serine proteases, can have neural inducing activity on ascidian ectoderms. In embryos of other species, although proteases are not regarded as candidates for the neural inducer, a few papers suggest the involvement of proteolysis in neural development. For example, it is reported that the dorsal lip has a higher catheptic activity than ventral tissue in the early gastrula of *Discoglossus pictus* (D'Amelio & Ceas, 1957) or *Xenopus laevis* (Deuchar, 1958), and that the injection of endopeptidase inhibitors into the dorsal region of *Xenopus*

concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml at the time of contact, that is, 2·5 h after isolation. Reduction of cytochalasin B facilitates contact (Okado & Takahashi, 1990*b*).

embryos causes abnormal gastrulation and defects of the eye and neural tube formation (Miyata & Kihara, 1987). However, in these reports they have stressed roles for proteolysis mainly in new protein synthesis during gastrulation and have not discussed the possibility of an involvement in neural inducing activity.

Possible mechanisms of neural induction by proteases

Since subtilisin mimicked the *in vivo* inducing activity and secretory process inhibitors, monensin, brefeldin A and bafilomycin A1, affected induction by contact, it is natural to have a working hypothesis that some inducing protease is secreted from the inducing $A_{4\cdot1}$ blastomere and that the released protease interacts with some receptor on the membrane of neurally competent blastomeres, producing intracellular signals to switch the cell fate (Fig. 7*A*).

Recently, genetic studies on *Drosophila melanogaster* development have disclosed that eleven maternal genes are involved in the specification of dorsal–ventral pattern at the beginning of development, i.e. the non-cellular or cellular blastoderm stage (DeLotto & Spierer, 1986). Of the eleven genes, *dorsal*, *snake*, *easter* and *toll* have been cloned and the primary structures of their encoded proteins have been clarified (Hashimoto, Hudson & Anderson, 1988; Steward, Zusman, Huang & Schedl, 1988; Chasan & Anderson, 1989). The embryos from mutant mothers, such as *dl/dl* females, develop in such a way that normal ventro-lateral pattern elements, which include presumptive neuroepithelial region, are displaced or absent and replaced by dorsal pattern elements (DeLotto & Spierer, 1986). Intensive molecular biological studies have shown that the *toll* gene appears to encode a transmembrane protein (Hashimoto *et al.* 1988) and both *snake* and *easter* genes seems to encode extracytoplasmic serine proteases (DeLotto & Spierer, 1986; Chasan & Anderson, 1989). These studies suggest that *toll* and *snake* and/or *easter* may interact each other to produce intracellular signals which will be transmitted to the *dorsal* protein, possibly one of the DNA binding proteins closely related to the avian cellular oncogene c-*rel* (Steward *et al.* 1988), and to direct the cells in the *dorsal*-activated part of the blastoderm to form ventral elements (Hashimoto *et al.* 1988). Thus, by analogy, it is highly probable that some serine protease, which is released, interacts with a membrane receptor, activating the receptor and resulting in neural induction of ascidian ectodermal blastomeres.

There are, however, many other possibilities which could explain the induction of ascidian blastomeres by proteases. Recently, Grunz & Tacke (1989) have shown that when *Xenopus laevis* blastula or early gastrula ectoderm is dissociated and the cells kept dispersed for up to 5 h prior to reaggregation, the resulting spheres of ectodermal cells differentiate autonomously into large neural structures independently of the neural inducer, while dissociated and immediately reaggregated ectoderm will only differentiate into ciliated epidermis, in accordance with the classical experiments. This autoneutralization can be prevented by cell supernatant from dissociated ectoderm, which contains extracellular matrix components (ECM) (Grunz & Tacke, 1990). The inhibiting substances contain a substantial amount of glyco-conjugated proteins, because the inhibitory effects are inactivated by phenol extraction (Grunz & Tacke, 1990). Although possible proteolysis of this inhibitory ECM was not discussed by the authors, limited degradation of the ECM around competent blastomeres of the ascidian embryo might be provoked by the applied

proteases, resulting in differentiation of the ectoderm blastomeres into neural derivatives. Since our way of isolating blastomeres with a glass needle was likely to keep the ECM intact, it is also possible that the release of degrading proteases occurs during induction by contact and that those proteases specifically destroy a local extracellular structure which normally suppresses the autonomous neural differentiation of competent ectodermal cells.

In the present experiments, the secretory process inhibitors, monensin, brefeldin A and bafilomycin A1, affected neural induction, allowing us to hypothesize that an inducer protease is secreted by inducing blastomeres (Fig. 7*A*). In fact, the secretion of serine proteases from the same *Halocynthia* eggs into the perivitelline space has been suggested to occur during fertilization or after activation by the ionophore A23187, since expansion of the perivitelline space just after egg activation is blocked by various serine protease inhibitors. Furthermore, the ranking of inhibitory potencies among inhibitors correlates well with those of the trypsin-like proteases purified from eggs (Sawada, Kawahigashi, Yokosawa & Ishii, 1985). This type of protease secretion has also been demonstrated in mouse eggs in the case of the breakdown of the zona *pellucida* glycoprotein (Moller & Wasserman, 1989). Thus, the blastomeres from the early 8-cell *Halocynthia* embryo may retain a mechanism for releasing proteases.

The roles of extracellularly released proteinases in cellular invasiveness, such as angiogenesis, tumour metastasis, wound healing and migration of cells during development, have been well documented (Mullins & Rohrlich, 1983), the importance being further demonstrated in the case of eversion of the insect imaginal disc (Pino-Heiss & Schubiger, 1989) and neurite growth of cultured neurones (Pittman, 1985). If a protease is an inducer in contact-mediated induction, the regulated proteolysis may be required to localize at the site of cell–cell contact. This could be achieved partly through membrane bound proteases and partly through a ubiquitous presence of protease inhibitors. The appearance of membrane-bound proteases has been reported during transformation of an embryonic fibroblast line (Chen & Chen, 1987), and also during degradation of serum amyloid protein A by mononuclear leukocytes (Zucker-Franklin, Lavie & Franklin, 1981). Recently, the physiological significance of membrane-bound subtilisin-type serine proteases has been well documented in the processing of prohormones, the proteases being defined as dibasic processing endopeptidase (Barr, 1991). Subtilisin and sublisin-like serine proteases have long been considered to be only of prokaryotic origin, but since the discovery of the KEX2 endoprotease as a subtilisin-like enzyme that cleaves the pro-α-factor, a mating hormone, in *Saccharomyces cerevisiae* (Mizuno, Nakamura, Oshima, Tanaka & Matsuo, 1988; Fuller, Brake & Thorner, 1989), several homologues of this enzyme have been cloned in mammals, including furin/PACE, which is known as a receptor-like protein, and the gene of which is located just ahead of a proto-oncogene (Bresnahan, Leduc, Thomas, Thorner, Gibson, Brake, Barr & Thomas, 1990). Although the subtilisin-like membrane-bound proteases are all suggested to be located in rough endoplasmic membranes or Golgi apparatus membranes (Barr, 1991), an interesting question arises as to whether the fact that subtilisin is much more effective than other proteases in the *Halocynthia* embryo is related to the activation of pro-receptors by dibasic processing endopeptidases bound to the surface membrane of the inducing blastomere. It should also be noticed as one of the

possible ways of activating receptors with proteolytic enzymes that Vu *et al.* (Vu, Hung, Wheaton & Coughlin, 1991) have demonstrated that a new amino-terminal of thrombin receptor created by cleavage with thrombin works as a tethered ligand and activates the receptor.

In order to prove the working hypothesis presented in the present paper, it is, however, necessary to identify the protease on the surface membrane of the inducing blastomere, $A_{4\text{-}1}$, at the time of induction and/or to identify a receptive element or elements on the membrane of the competent blastomere, $a_{4\text{-}2}$, that is common to both induction by proteases and induction by contact.

Early determination of neural competence

The anterior half of the ectoderm has a larger presumptive region for neural tissues than the posterior half (Conklin, 1905; Nishida, 1987). It is questioned whether this regional specificity in neural development depends entirely on the regional difference in competence of the blastomeres to be induced. In our previous report, a regional difference of neural competence was demonstrated between anterior and posterior ectodermal blastomeres in 8-cell embryos (Okado & Takahashi, 1990*b*) and, in the present experiment, among four ectodermal blastomeres in the 16-cell embryo. These studies revealed that anterior blastomeres in ectoderm were neurally induced with higher frequency than posterior blastomeres (Fig. 6*A* and *B*). This tendency is consistent with the more neural lineage in the anterior half of the ectoderm reported by Nishida (1987) in normal development, as shown in Fig. 6*C*. Therefore, the regional specificity of neural development *in vivo* is largely derived from the regional difference of neural competence among ectodermal blastomeres. Since the ascidian egg has long been considered as a typical mosaic one (Conklin, 1905), it is claimed that the apparent inductive formation of neural tissues is not a real one but simply results from evocation of a predetermined neural lineage (Reverberi, 1971). Our present results appear to confirm this claim by demonstrating the importance of predetermination of the neural competence ahead of induction. However, in order to discuss the issue completely, the regional difference of inducing activity among inducing blastomeres is also required to be examined. Further, although the $b_{5\text{-}4}$ blastomere has no neural fates *in vivo* (Nishida, 1987), it was induced to become a neural-type cell by treatment with subtilisin or by contact with the $A_{4\text{-}1}$ blastomere, switching the developmental fate from epidermal to neural. This discrepancy is most probably due to inaccessibility of $b_{5\text{-}4}$ derivatives to the derivatives of $A_{4\text{-}1}$ in the normal embryo during the gastrula stage. In this case the morphogenetic movement of inducer blastomeres relative to competent blastomeres *in vivo* could be considered to be the cause of regional specificity. It is thus concluded that induction actually occurs in the ascidian embryo and that the difference in predetermined competence is not the only factor underlying regional specificity in neural development, as shown in other vertebrate embryos.

We acknowledge the Marine Biology Laboratory of Thohoku University at Asamushi, Miyagi Prefectual Fisheries Experimental Station at Kesennuma, Professor K. Suzuki in the Department of Fisheries of Kitazato University at Sanriku, and Wakkanai City Aquarium for collecting materials. We thank Professor Y. Mitsui (Nagaoka Institute for Technological Sciences), Professor

Emeritus S. Murao (Osaka Prefectural University) and Professor Emeritus S. Ishii (Hokkaido University) for providing us with SSI. We thank Dr Moriyama (Institute of Scientific and Industrial research, Osaka University) for providing bafilomycin A1. We thank Dr Harumasa Okamoto of our Department of Neurobiology for his valuable suggestions during present experiments and Dr Yasushi Okamura for his comments on the manuscript. This investigation was supported by Grants-in-Aid for scientific research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

BARR, P. J. (1991). Mammalian subtilisins: The long-sought dibasic processing endoproteases. *Cell* **66**, 1–3.

BRESNAHAN, P. A., LEDUC, R., THOMAS, L., THORNER, J., GIBSON, H. L., BRAKE, A. J., BARR, P. J. & THOMAS, G. (1990). Human *fur* gene encodes a yeast KEX2-like endoprotease that cleaves pro-β-NGF *in vivo*. *Journal of Cell Biology* **111**, 2851–2859.

CHASAN, R. & ANDERSON, K. V. (1989). The role of *easter*, an apparent serine protease, in organizing the dorsal-ventral pattern of the Drosophila embryo. *Cell* **56**, 391–400.

CHEN, J.-M. & CHEN, W.-T. (1987). Fibronectin-degrading proteases from the membranes of transformed cells. *Cell* **48**, 193–203.

CONKLIN, E. G. (1905). The organization and cell-lineage of the ascidian egg. *Journal of the Academy of Natural Sciences of Philadelphia* **13**, 1–119.

D'AMELIO, V. & CEAS, M. P. (1957). Distribution of protease activity in blastula and early gastrula of *Discoglossus pictus*. *Experientia* **XIII/4**, 152–153.

DELOTTO, R. & SPIERER, P. (1986). A gene required for the specification of dorsal–ventral pattern in Drosophila appears to encode a serine protease. *Nature* **323**, 688–692.

DEUCHAR, E. M. (1958). Regional differences in catheptic activity in *Xenopus laevis* embryos. *Journal of Embryology and Experimental Morphology* **6**, 223–227.

FULLER, R. S., BRAKE, A. J. & THORNER, J. (1989). Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246**, 482–486.

GURDON, J. B. (1987). Embryonic induction – molecular prospects. *Development* **99**, 285–306.

GRUNZ, H. & TACKE, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differentiation and Development* **28**, 211–218.

GRUNZ, H. & TACKE, L. (1990). Extracellular matrix components prevent neural differentiation of disaggregated Xenopus ectoderm cells. *Cell Differentiation and Development* **32**, 117–124.

HAMBURGER, V. (1988). *The Heritage of Experimental Embryology: Hans Spemann and the Organizer*. Oxford University Press, New York.

HARPEL, P. C. (1976). Human α2-macroglobulin. *Methods in Enzymology* **XLV**, 639–652.

HASHIMOTO, C., HUDSON, K. L. & ANDERSON, K. V. (1988). The *toll* gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269–279.

HIRANO, T., TAKAHASHI, K. & YAMASHITA, N. (1984). Determination of excitability types in blastomeres of the cleavage-arrested but differentiated embryos of an ascidian. *Journal of Physiology* **347**, 301–325.

LIPPINCOTT-SCHWARTZ, J., YUAN, L. C., BONIFACINO, J. S. & KLAUSNER, R. D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801–813.

MISUMI, Y., MISUMI, Y., MIKI, K., TAKATSUKI, A., TAMURA, G. & IKEHARA, Y. (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *Journal of Biological Chemistry* **261**, 11398–11403.

MIYATA, S. & KIHARA, H. K. (1987). Effects of endopeptidase inhibitors on gastrulation of Xenopus eggs. *Development, Growth and Differentiation* **29**, 427–432.

MIZUNO, K., NAKAMURA, T., OHSHIMA, T., TANAKA, S. & MATSUO, H. (1988). Yeast KEX2 gene encodes an endopeptidase homologous to subtilisin-like serine proteases. *Biochemical and Biophysical Research Communications* **156**, 246–254.

MOLLER, C. C. & WASSARMANN, P. M. (1989). Characterization of a proteinase that cleaves *zona pellucida* glycoprotein ZP2 following activation of mouse eggs. *Developmental Biology* **132**, 103–112.

MULLINS, D. E. & ROHRLICH, S. T. (1983). The role of proteinases in cellular invasiveness. *Biochimica et Biophysica Acta* **695**, 177–214.

NARAHASHI, Y. (1970). Pronase. *Methods in Enzymology* **XIX**, 651 664.

NISHIDA, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Developmental Biology* **121**, 526 541.

NISHIDA, H. & SATOH, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Developmental Biology* **132**, 355–367.

OKADO, H. & TAKAHASHI, K. (1988). A simple "neural induction" model with two interacting cleavage-arrested ascidian blastomeres. *Proceedings of the National Academy of Scienes of the USA* **85**, 6197–6201.

OKADO, H. & TAKAHASHI, K. (1990*a*). Differentiation of membrane excitability in isolated cleavage-arrested blastomeres from early ascidian embryos. *Journal of Physiology* **427**, 583–602.

OKADO, H. & TAKAHASH, K. (1990*b*). Induced neural-type differentiation in the cleavage-arrested blastomere isolated from early ascidian embryos. *Journal of Physiology* **427**, 603–623.

ORTOLANI, G., PATRICOLO, E. & MANSUETO, C. (1979). Trypsin-induced cell surface changes in ascidian embryonic cells. Regulation of differentiation of a tissue specific protein. *Experimental Cell Research* **122**, 137–147.

PINO-HEISS, S. & SCHUBIGER, G. (1989). Extracellular protease production by Drosophila imaginal discs. *Developmental Biology* **132**, 282–291.

PITTMAN, R. N. (1985). Release of plasminogen activator and calcium-dependent metalloprotease from cultured sympathetic and sensory neurones. *Developmental Biology* **110**, 91–101.

REVERBERI, G. (1971). Ascidians. In *Experimental Embryology of Marine and Fresh-water Invertebrates*, ed. REVERBERI, G., pp. 507–550. Elsevier, Amsterdam/New York.

ROSE, S. M. (1939). Embryonic induction in the Ascidia. *Biological Bulletin* **77**, 216–232.

SAWADA, H., KAWAHIGASHI, M., YOKOSAWA, H. & ISHII, S. (1985). Trypsin-like enzyme from eggs of the ascidian (protochordate), *Halocynthia roretzi*. Purification, properties, and physiological role. *Journal of Biological Chemistry* **260**, 15694–15698.

SCHROEDER, T. E. (1978). Cytochalasin B, cytokinesis, and the contractile ring. In *Cytochalasins: Biochemical and Cell Biological Aspects*, ed. TANENBAUM, S. W., pp. 91–112. North-Holland, Amsterdam.

SPEMANN, H. (1938). *Embryonic Development and Induction*, pp. 141–198. Yale University Press, New Haven, CT, USA.

STEWARD, R., ZUSMAN, S. B., HUANG, L. H. & SCHEDL, P. (1988). The dorsal protein is distributed in a gradient in early Drosophila embryos. *Cell* **55**, 487–495.

TARTAKOFF, A. M. (1983). Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**, 1026–1028.

TONOMURA, B., SATO, S., MIWA, M. & KOMIYAMA, T. (1985). General characterization of SSI. In *Protein Protease Inhibitor – The Case of Streptomyces Subtilisin Inhibitor*, ed. HIROMI, K., AKASAKA, K., MITSUI, Y., TONOMURA, B. & MURAO, S., pp. 139–157. Elsevier, Amsterdam, New York.

UMATA, T., MORIYAMA, Y., FUTAI, M. & MEKADA, E. (1990). The cytotoxic action of diphtheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase. *Journal of Biological Chemistry* **265**, 21940–21945.

VU, T.-K. H., HUNG, D. T., WHEATON, V. I. & COUGHLIN, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057 1068.

WHITTAKER, J. R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. *Proceedings of the National Academy of Sciences of the USA* **70**, 2096–2100.

WHITTAKER, J. R. (1987). Neural induction in ascidian embryos *redivivus*. *Biological Bulletin* **173**, 428–429.

ZUCKER-FRANKLIN, D., LAVIE, G. & FRANKLIN, E. C. (1981). Demonstration of membrane-bound proteolytic activity on the surface of mononuclear leukocytes. *Journal of Histochemistry and Cytochemistry* **29**, 451 456.

stem cells in culture.
SO SCIENCE, (1994 Aug 19) 265 (5175) 1098-101.
Journal code: UJ7; 0404511. ISSN: 0036-8075.

L15 ANSWER 10 OF 11 MEDLINE
AU Okado H; Takahashi K
TI Neural differentiation in cleavage-arrested ascidian blastomeres induced by a proteolytic enzyme.
SO JOURNAL OF PHYSIOLOGY, (1993 Apr) 463 269-90.
Journal code: JQV; 0266262. ISSN: 0022-3751.

L15 ANSWER 11 OF 11 MEDLINE
AU Klein B Y; Gal I; Segal D
TI Selection of malonate-resistant stromal cell-derived osteoprogenitor cells in vitro.
SO JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 190-7.
Journal code: HNF; 8205768. ISSN: 0730-2312.

Shin-Lin Chen
AU 1633
CM1 12A15
Mail Box # 12E12
(703)305-1678

11/23
09686880

Journal of Cellular Biochemistry 51:190–197 (1993)

Selection of Malonate-Resistant Stromal Cell-Derived Osteoprogenitor Cells In Vitro

Benjamin Y. Klein, Irena Gal, and David Segal

Laboratory of Experimental Surgery, Department of Orthopedic Surgery, Hadassah Medical Center, Ein-Kerem Jerusalem 91120, Israel

Abstract Bone marrow stromal cells give rise to osteoprogenitor cell (OPC) colonies, with characteristic mineralized bone nodules in vitro. During differentiation, OPCs in the culture are surrounded by heterogeneous populations of various cell lineages and by different OPC differentiation stages. In the present study, attempts were made to increase the homogeneity of OPCs in culture. The reliance on energy metabolism restricted to glycolysis, which is specific to the premineralizing skeletal cells, was tested as a selectable marker for cells in this stage. Day 12 alkaline phosphatase (ALP) and day 20–21 calcium precipitates were used as early and late OPC differentiation markers. Malonate, a competitive inhibitor of succinate dehydrogenase, was added to the OPC stimulation medium, to interfere with the Krebs cycle-dependent energy metabolism operating in most of the stromal cells. OPCs that entered the stage of energy metabolism restricted to glycolysis were expected to become malonate resistant. Malonate showed dose and time dependence, 10 mM malonate added on day 3, decreased day 12 ALP activity/well to the lowest level. Variations in time and length of exposure to malonate used during the first 12 days of differentiation showed an inverse correlation between specific ALP activity and cell yield. Malonate-treated variations of specific ALP and of cell yield indices were up to 30- to 40-fold larger than variations within day 21 calcium precipitates. Thus, calcifying cells were almost unchanged relatively to noncalcifying cells. These results indicate that malonate-resistant cells are mostly selected, rather than induced, to differentiate by malonate. The results also show that stromal derived OPCs undergo a similar biochemical stage as in chondrocytes.

Key words: energy metabolism, glycolysis, differentiation stage, alkaline phosphatase, mineralization

During differentiation, skeletal cells undergo a transient biochemical alteration, their microenvironment becomes hypoxic [Brighton and Heppenstall, 1971], and their redox state is changing [Shapiro et al., 1982]. During hypoxia observed at the growth plate microenvironment, the chondrocytes rely on glycolysis as a source of energy. This biochemical alteration is characteristic of a differentiation stage in these cells and should provide an opportunity to enrich selectively osteoprogenitor cells (OPCs) at their "anaerobic" stage relative to the surrounding majority of non-OPCs that use more energy from "aerobic" metabolism. Hypothetically, at their anaerobic glycolytic stage, unlike other cells, OPCs should be selectively resistant to blockers of the "postglycolytic" pathway.

Received August 21, 1992; accepted September 23, 1992.

Address reprint requests to Benjamin Y. Klein, Laboratory of Experimental Surgery, Department of Orthopedic Surgery, Hadassah Medical Center, Ein-Kerem Jerusalem 91120, Israel.

The aerobic energy metabolism depends on reactions catalyzed by mitochondrial enzymes of the citric acid cycle. Therefore diminishing the citric acid cycle efficiency by blocking key enzymes is expected to arrest cell proliferation and cellular functions of non-OPCs or OPCs in their aerobic stage.

The mitochondria, in which the citrate acid cycle occurs, also participates in controling the cellular calcium metabolism in both soft and hard tissues. The mitochondria in various soft tissues accumulates calcium [Carafoli and Sottocasa, 1984] by a metabolism-dependent process, but mitochondria in skeletal cells presumably have an additional mechanism for calcium binding [Shapiro and Lee, 1975]. It was also found that calcium precipitation in the growth plate is preceded by calcium efflux from mitochondria [Brighton and Hunt, 1978]. Chondrocytic mitochondria accumulates calcium in a crystallized form [Lehninger, 1970], which is released when cartilage mineralization takes place. The mechanism whereby calcium is ex-

truded from skeletal cell mitochondria has not been completely determined yet, but studies in the past have elucidated part of this process. It is known for long that cell under low oxygen tension restrict their energy utilization to the premitochondrial glucolytic pathway (anaerobic metabolism) [Krebs, 1953]. A shift to glycolytic metabolism has indeed been observed between the proliferative zone and the hypertrophic condrocytes before entering to the mineralizing front in the growth plate [Brighton et al., 1969]. Under anaerobic conditions, molecular oxygen is less available in the mitochondria to cooperate with oxidative phosphorylation and extrusion of NADH-derived protons. Accordingly, it has been found that under anaerobic conditions, in the hypertrophic chondrocytes of the growth plate, the NADH/NAD ratio is elevated [Shapiro, 1982]. Earlier it was found that a lower cytosolic pH and accumulation of phosphoenol pyruvate, both characteristic of a glycolytic anaerobic metabolism, are associated with calcium release from chondrocytic mytochondria [Shapiro and Lee, 1978]. Also the stroma derived OPCs presumably undergo a stage with characteristics of anaerobic metabolism during stromal cell differentiation. Stromal cells constitute a heterogeneous population [Owen et al., 1987]; furthermore, OPCs themselves at any time in culture constitute a heterogeneous population [Rodan et al., 1988]. In order to analyse a homogeneous population of a single OPC differentiation stage, it is necessary to enrich the cell population with this particular stage. In the present publication, we describe the effect of sodium malonate, a competitive inhibitor of succinate dehydrogenase, on the relative enrichment of stromal cell-derived OPCs in culture.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, sodium malonate, dexamethasone, ascorbate, β-glycerophosphate, Alizarin Red S, Light Green SF Yellowish were purchased from Sigma, St. Louis, MO. Fetal calf serum (FCS) was purchased from Grand Island Biological Company, N.Y.

Stromal Cell Culture

Bone marrow cell suspensions obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25-cm^2 flasks, 10^8 cells/flask. The stroma cells were obtained as described by [Maniatopoulos et al., 1988], removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium, that consisted of DMEM (Dulbecco modified Eagle's medium) supplemented with 15% fetal calf serum and antibiotics, in a humid 10% CO_2 atmosphere. For the experimental cultures stroma cells were removed 2 weeks later by trypsinization and were plated in microtiter plates, 5,000 cells/well and grown in osteoprogenitor cell (OPC) stimulation medium. This consisted of maintenance medium containing 10^{-8} M dexamethasone, 50 μg ascorbate/ml and 10 mM β-glycerophosphate.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. ALP activity in primary bone cell cultures was shown to peak between days 10 and 12 [Deren et al., 1990]; therefore, day 12 of dexamethasone stimulation was set for ALP assay and cell count. Growth medium was removed, and the cells were washed twice in situ with 0.2 ml TNC (50 mM Tris, 150 mM NaCl pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TNC, 1.33 mg/ml, was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min, and the optical density of the hydrolysed pNPP was measured in a multichannel spectrophotometer at 405-nm wavelength. ALP activity was expressed either as nMol/time/cell (or per well) or as O.D. units. For experiments that required propagation of the same cells in culture, after undergoing an in situ ALP assay, the procedure was carried out under sterile conditions. A translucent sterile coverslip was placed on the plates during the spectrophotometry. Immediately following O.D. determination the substrate was replaced by growth medium, after the cells were washed once with TNC, to continue their propagation in culture.

For a study of the levamisole effect on stromal differentiation (submitted), it was necessary to determine whether in situ ALP activity at pH 7.6 correlates with the activity at pH 10.3. The assay at both pH conditions was performed in tandem in each plate. The linear regression for cells grown in OPC inductive medium showed a direct correlation ($r = 0.839$) between phosphatase assays at both pH conditions, this is acceptable considering the nature of such a cellular assay. The linear regression of the enzyme activities in cells cultivated in the presence of le-

vamisol, showed an even better correlation ($r = 0.919$). This result indicates that 90-min incubation at pH 7.6 is adequate for comparative ALP assays if groups with multiple samples are being compared.

Quantitative Cell Staining

After the ALP assay, in some of the experiments, cells were stained using the methylene blue (MB) method [Goldman and Bar-Shavit, 1979]. The cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O, and air-dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. The O.D. of the eluted MB was measured at 620 nm by a multichannel spectrophotometer; 1.0 unit is equivalent to 5×10^4 stromal cells.

Measurement of In Vitro Precipitated Calcium

After 2 weeks of culture in OPC stimulation, medium calcium precipitates began to appear around some of the stroma cells, demonstrable by Alizarin Red as an orange-red staining. To quantitate these precipitates plates were washed twice with TNC and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations. The precipitates were expressed as μg Ca/well.

Alizarin Red S Staining

For a semiquantitative demonstration of mineralization, 20- to 21-day-old stimulated cultures were fixed 20 min in undiluted methanol. Subsequently 1.0 g Alizarin sodium sulfate (Sigma A-3757) in an aqueous solution of 0.1% NH_4OH was applied to the cultures for 2 min and then washed 3 times with distilled water. The cultures were counter stained for 2 min with 1% Light Green SF Yellowish (Sigma L-5382) in 1% acetic acid. Cultures were air-dried after rinsing with distilled water.

RESULTS

Determination of ALP Activity During Stromal Cell Differentiation

Before testing the ability of malonate to affect stromal cell commitment to day 21 mineralization (as related to ALP activity), determination of the appropriate day for ALP assay was required. Stromal cells were cultured with OPC stimulation medium containing dexamethasone, ascorbate, and βGP. ALP activity and cell

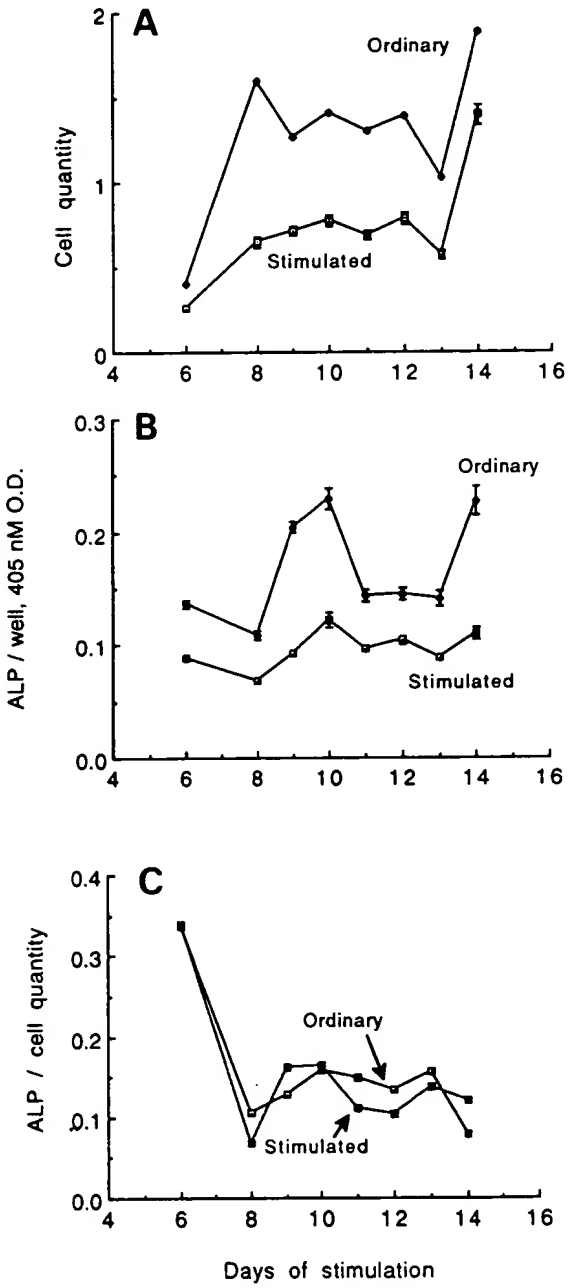


Fig. 1. Effect of OPC stimulation medium on stromal cell quantity, on ALP activity, and on specific ALP activity. On day (−)3, microtiter wells were seeded with 3×10^3 cells/well and on day 0 the medium was changed to OPC stimulation medium. ALP activity was measured and the methylene blue cell count was performed at several time intervals, a separate plate was assigned to each time point. Each point represents the mean ±SE of 20 microtiter wells.

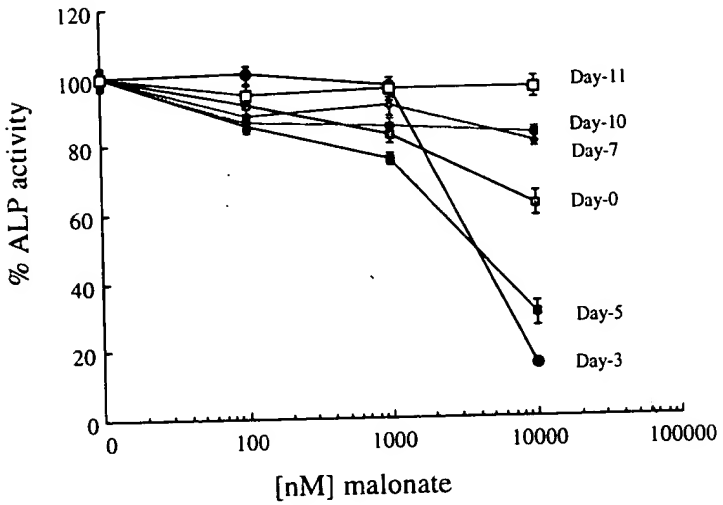


Fig. 2. Malonate effect on day 12 ALP activity in stimulated stromal cells. Stromal cells were grown in OPC stimulation medium and in different concentrations of malonate (from 10.0 mM to 100 nM). Malonate was added to the cultures at different days (as indicated near each curve) and removed on day 12. ALP activity was measured on day 12. Each point represents the mean ±SE of 24 wells.

quantitation were performed during differentiation at different time intervals. Figure 1A,B shows that the stimulation medium diminishes the absolute ALP activity and cell quantity, respectively. The specific ALP activity (Fig. 1C) expressed per cell on day 9 was only 25% higher, and on day 12 it was even lower than in the control cultures. The specific ALP activity in this system is not a net representative of all ALP-expressing osteogenic cells. This is because not all the osteogenic cells show an increased ALP activity at every instant during the differentiation period; also, some cells not associated with mineralization express ALP [Kamalia et al., 1992]. However, it is reasonable that ALP activity in a greater portion of the stimulated cultures is attributed to high ALP-expressing osteogenic cells, compared with the unstimulated cultures. Conversely, a diminishing portion of ALP activity is attributed to nonosteogenic cells. The OPC stimulation medium induces increased mineralization by increasing the number of osteogenic cell colonies. Calcium precipitation is detected in our system from day 14; thereafter (data not shown), this timing was also demonstrated in cultured rat calvarial osteoblasts [Pockwinse et al., 1992].

ALP Response to Malonate

Sodium malonate was added at different concentrations and time intervals, to several stromal cell cultures grown with OPC stimulation medium. The addition of malonate (Fig. 2) resulted in a low day 12 ALP activity; the dose with the highest effect on absolute ALP/well was 10 mM, being most prominent when added on day 3. Day 12 was chosen for ALP assays in this experiment based on its elevation in studies on osteogenic cells [Deren et al., 1991]. The diminished ALP activity was not due to inhibition of enzyme function, but rather to a diminished day 12 cell population observed by microscopy (Fig. 5A).

Correlation Between Day 21 Calcium Precipitate With Earlier ALP Activity

Days 20–21 were chosen for measuring cell-mediated calcium precipitates, because at that time they are detectable by Alizarin staining and by atomic absorption. Previous studies on stromal cell mineralization inhibition under the continuous presence of levamisole (submitted) suggested that inhibition of day 21 mineralization has at least two components, one consists of inhibition of enzymatic activity during crystal growth (a late stage controlled by ALP), the other component is an early inhibitory effect induced by levamisole during the first week in culture, before mineralization takes place. Therefore, the present study required determination of appropriate days during differentiation in which the ALP activity shows the best correlation with day 21 mineralization. For this pur-

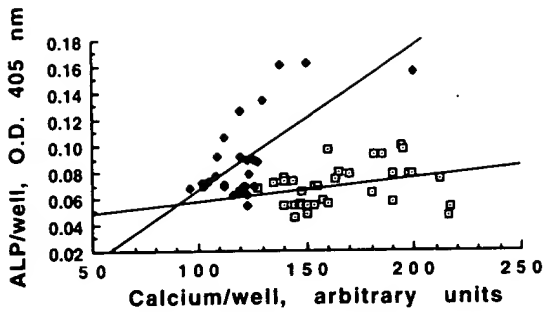


Fig. 3. Correlations between day 12 ALP activity and day 21 calcium precipitation of malonate-treated and untreated stroma. Stromal cells were cultured from day 0 with OPC stimulation medium in the presence (empty squares) and absence (closed diamonds) of 10 mM malonate, which was added on day 3 and removed on day 12. ALP assay was performed on day 12 under sterile conditions at pH 7.6; subsequently, the cells of both groups were cultivated in OPC stimulation medium until day 21. The correlation coefficient of malonate treated and untreated cultures is r = 0.295 and 0.670, respectively.

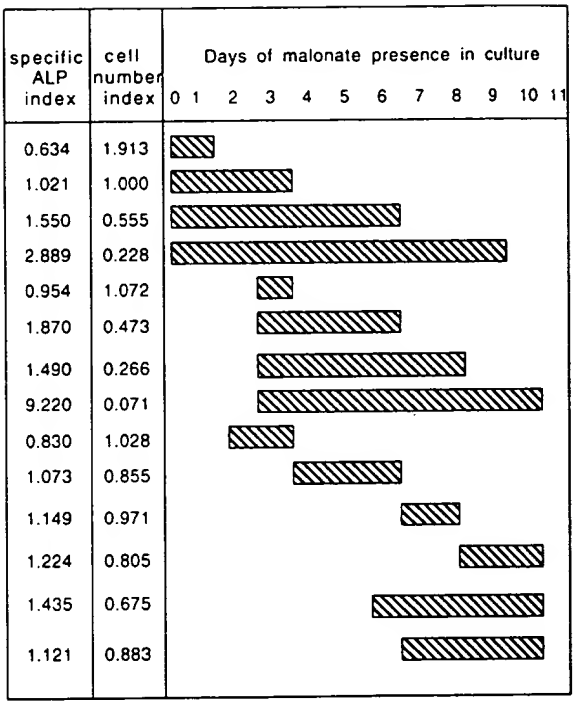


Fig. 4. Disection of the effect of malonate on stromal cells during the first 11 days of differentiation and proliferation. Stromal cells were stimulated with OPC stimulation medium from day 0 and malonate was added on different days and removed on different days. On day 11, specific ALP activity and cell quantity were determined and ratios of the values of cells cultivated with/without malonate are expressed as specific ALP index and cell No. index. For the mean of each ratio, n = 20.

pose, stromal cells were stimulated in culture and individual wells of 3 plates underwent in situ ALP assay, each on a different day (days 6, 9, and 12). Malonate was added to one-half of the wells in each of these plates, for a final concentration of 10 mM on day 3, as inferred from the dose response presented in Figure 2. Malonate was removed before the ALP assay and was not returned thereafter. Immediately after the ALP assay, the cells continued to grow in OPC stimulation medium (without malonate) and on day 21, calcium precipitates were measured. Figure 3 illustrates the regression lines of malonate-treated and untreated cultures of stromal cells grown in OPC stimulation medium. Each point represents day 12 ALP plotted against day 21 calcium of the same individual well. One of the two linear regressions in Figure 3 (the line with the lesser slope) represents malonate-treated cells. In the cultures without malonate, the correlation of day 21 Ca with day 12 ALP activity (r = 0.67) is better than with day 6 and 9 ALP activity (r = 0.044 and 0.219, respectively). It is also slightly better than the correlation with ALP of day 21 (where r was close to 0.6). These results justify the use of day 12 ALP activity as a differentiation marker that relates to cell-mediated calcification measured on day 21. Day 12 ALP in this study fulfills a function of a differentiation marker, independent of the direct enzymatic role in calcification played by ALP on day 21.

The Effect of Malonate on the Differentiating Stromal Cell Population

To determine the time ranges during differentiation at which malonate has the highest positive effect on osteoprogenitor cell (OPC) yield and the highest negative effect on non-OPC yield, 10 mM malonate was added and removed from the cultures at different days. OPC differentiation was determined on day 12, by measuring ALP activity/cell (specific ALP). Figure 4 illustrates various time ranges at which the cultures were exposed to 10 mM malonate, as well as its effect on OPC differentiation and on stromal cell proliferation. Results are expressed as the ratio (index) between malonate-treated and untreated controls. The lowest OPC yield (specific ALP index = 0.634) was associated with the highest cell proliferation (cell No. index = 1.913) and was obtained when cultures were exposed to malonate only on days 0–1 (upper line in Fig. 4). The increase in OPC yield was directly related to the duration of the expo-

sure to malonate. When malonate was added on day 3 of OPC stimulation and maintained only for 1 day, a higher OPC yield was observed (specific ALP index = 0.954), and it was associated with a lower cell proliferation (cell No. index = 1.072). The highest OPC yield (specific ALP index = 9.22) was obtained when malonate was added on day 3 of stromal OPC stimulation and maintained up to day 11; this was associated with the lowest cell yield compared to other cultures (cell No. index = 0.071).

TABLE I. Comparison Between Effects of Malonate on Day 21 Calcium Precipitation With the Effects on the Differentiated OPC Yield (n = 20)

Day malonate added to culture	Day 0	Day 3	Day 5	Day 7
Day 21* calcium index	1.202	0.976	1.148	1.209
Day 12 cell no. index	0.228	0.071	0.675	0.883
Day 12-specific ALP index	2.889	9.220	1.435	1.121

*Malonate removed on day 12.

The Early Malonate Effect on Day 21 Cell-Mediated Mineralization

Malonate was added to differentiating stromal cells on day 0 from the start of OPC stimulation for a 12-day exposure and at three different time intervals, with diminishing length of exposure. Malonate was removed from the cultures on day 12; after washing the cells with TNS, they were cultivated in OPC stimulation medium until Day-21 for quantitation of acid-soluble calcium precipitates. Table I shows the calcium index/well aligned with indices of specific ALP and cell No./well of proper malonate exposure durations, taken from Figure 4. Calcium precipitates

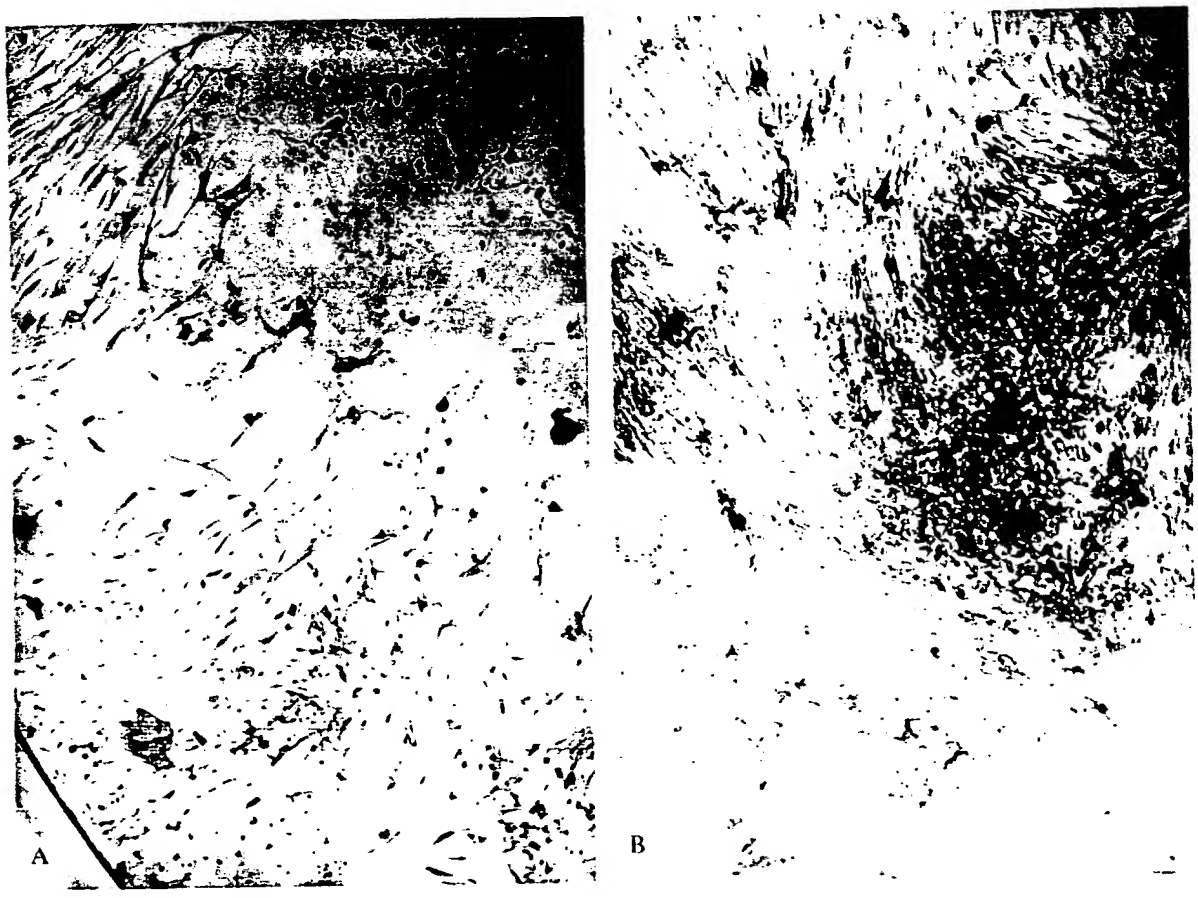


Fig. 5. Alizarin Red staining of day 21 calcium precipitates in stromal cell cultures cultivated in OPC stimulation medium. A: Cells were grown in 10 mM malonate from day 3 (A) to day 9 (see Fig. 4, line 7). B: Controls were grown without malonate. Cells in the regions of relatively dense cellularity were counted, and the ratio of the mean counts from 5A and 5B is comparable to the cell No. index on day 12 (see text). ×100.

sustained minor changes in the presence of malonate relative to the major changes in the specific ALP/well and in cell quantities/well. This is consistent with the hypothesis that malonate selectively arrested the proliferation of non-OPCs but permitted the growth or survival of OPCs.

The most prominent (1400%) variable seen at the presence of malonate was the cell No. index observed when malonate was added on day 3, it was associated with a minimal effect (20–30%) on day 21 calcium index. This suggests that, in the cultures, on day 3, there is a peak of malonate-resistant OPCs that can tolerate malonate for up to 9 days, probably under continuous accumulation of such newly differentiated OPCs.

Microscopic View of Malonate Effect on Stromal Cell Quantity

Two opposing wells, one treated with malonate from day 3 for 6 days. The other untreated controls (Fig. 5A,B, respectively) were stained with Alizarin Red and with Light Green on day 21. In both wells there are regions with dens cell growth and regions with lower cell density. In the malonate-treated culture (Fig. 5A), the "crowded" regions contained 10.105 ± 3.11 cells/cm^2, whereas in the control (5b), they contained 47.555 ± 7.689 cells/cm^2. The ratio between these cell counts is 0.212, close to the cell No. index of 0.266 of the appropriate sample in Figure 4 (line 7).

DISCUSSION

In the present work, we examined the effect of malonate on the proliferation of stromal cells and on their differentiation in vitro. Based on studies with differentiating bone cells, ALP quantitation was performed on day 12. Dose-response analysis showed that 10 mM malonate is the appropriate concentration for lowering the absolute ALP activity/well on day 12. The diminished absolute ALP activity was later found to reflect a diminished stromal cell number and the cells even showed increased specific ALP activity/cell contrary to the decreased absolute activity/well.

In nonstimulated stromal cell cultures, specific ALP activity peaks were observed on days 6, 9, and 12, corresponding to days 3, 6, and 9 of cultures grown in dexamethasone-containing osteoprogenitor cell (OPC) stimulation medium. In dexamethasone-stimulated cultures, regression analysis between ALP activity/well (on days 6, 9, and 12) and day 21 precipitated-calcium revealed that the best correlation was obtained between day 12 ALP activity and day 21 calcium. These results indicated that, in differentiating stromal cells as well, day 12 is appropriate for the performance of the ALP assay and cell counting, if day 21 calcium results should be related to ALP. In stimulated stromal cells, calcium precipitates appear after 15 days in culture, but they become prominent on day 20 (data not shown). Therefore, calcium was always measured on days 20–21, although calcium may accumulate on bone nudules at least until day 35. This is in accord with results in calvarial osteoblasts [Pockwinse et al., 1992], which showed that 30% of maximal ALP expression and 30% of maximal mineralization occur on days 12 and 20, respectively.

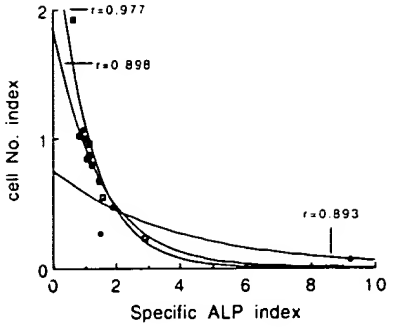


Fig. 6. Inverse correlation between relative values of malonate-selected stromal cell count and the relative specific ALP activity in selected cells. Exponential regression lines of three separate groups of results taken from Figure 4. Open squares represent malonate added on day 0 and removed on different days, r = 0.977 (4 upper lines in Fig. 4). Closed diamonds represent malonate added on day 3 and removed on different days thereafter, r = 0.893 (second four lines in Fig. 4). Closed squares represent mostly samples of 2–3 days of exposure, r = 0.898 (last six lines in Fig. 4). Each point represents the correlation between the mean of 20 index values. Each index value represents the ratio between specific ALP (or cell count) in one well of malonate treated and its proper control well on the same plate.

The various periods and the points of entry at which malonate was administered to the cultures during the first half of stromal cell differentiation revealed an inverted relation between specific ALP activity and total stromal cell count, both performed on day 12. Exponential regression lines (Fig. 6) obtained from three different groups of results, shown in Figure 4, show a sufficiently high correlation between these two parameters. The inverted correlation between differentiation (specific ALP) and proliferation (cell No.) in stromal cells is similar to the find-

TABLE II. Lack of Day 3 Malonate Ability to Substitute for Dexamethasone (DX) and Ascorbate (VC), as Mineralization Inducer in Str mal Cells

Culture no.	Ingredients in the OPC stimulation medium							Day 21 alizarin red staining
	Days 0–3		Days 3–12			Days 12–21		
	DX + VC	βGP[a]	DX + VC	βGP	Malonate	DX + VC	βGP	
1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(4+)
2	(+)	(+)	(−)	(+)	(+)	(−)	(+)	Trace
3	(−)	(+)	(−)	(+)	(+)	(−)	(+)	(3+)
4	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)

[a]β-Glycerophosphate.

ings shown in differentiating osteoblasts [Stein et al., 1990]. Figure 6 shows that malonate treatment complys with and does not abrogate this inverted ratio.

The relative enrichment in OPCs caused by a longer presence of malonate is consistent with a selective effect of malonate favoring the OPC population over non-OPCs. This is supported by the fairly constant mineral quantities associated with different malonet entries and length of exposure. Based on the minimal changes in calcium, it is likely that malonate has mainly arrested the growth of non-OPCs with a weaker effect on the OPC population.

The experiments represented in Figures 2 and 4 suggest that the day of malonate addition also influences the relative enrichment of the cultures with ALP-expressing OPCs. Day 3 differs from other days by the stromal cell response to malonate. Malonate is most efficient on day 3 in enriching the cultures with OPCs of high ALP specific activity, as measured on day 12. Unexpectedly, adding malonate on day 3 is even better than adding it on day 0. The mechanism for this phenomenon is not clear; however, it can be used to test the conclusion that malonate has a selection rather than an induction effect on OPCs. Confirmation that the role played by malonate is restricted mainly to OPC selection was achieved by showing that ingredients in the stimulation medium are responsible for the induction of this phenomenon and that they can not be substituted by malonate. Table II shows that when dexamethasone and ascorbate are used for OPC stimulation starting on day 0, they must be maintained beyond day 3, since their removal on day 3 will result in a lack of day 21 mineralization in spite of the presence of malonate from day 3 to day 12. Interestingly β-glycerophosphate was sufficient to induce mineralization on its own in the presence of malonate, but it failed to induce it if dexamethasone and ascorbate were administered on day 0 but discontinued on day 3. These results indicate that day 3 malonate can select, on day 12, for cells that are prepared to mineralize later within several days.

REFERENCES

Brighton CT, Ray R, Soble L, Kuettner K (1969): J Bone Joint Surg 51A:1383–1412.

Brighton CT, Heppenstall RB (1971): J Bone Joint Surg 53A:719–728.

Brighton CT, Hunt RM (1978): Metab Bone Dis Relat Res 1:199–204.

Carafoli E, Sottocasa G (1984): In Ernester L (ed): "Bioenergetics." pp 269–289.

Deren JA, Kaplan FS, Brighton CT (1990): Clin Orthop 252:307–312.

Goldman R, Bar-Shavit Z (1979): JNCI 63:1009–1016.

Kamalia N, McCulloch CAG, Tenenbaum HC, Limback H (1992): Blood 79:320–326.

Krebs HA (1953): Br Med Bull 9:97–104.

Lehninger AL (1970): Biochem J 119:129–138.

Maniatopoulos C, Sodek J, Melcher AH (1988): Cell Tissue Res 254:317–330.

Owen ME, Cave J, Joyner CJ (1987): J Cell Sci 87:731–738.

Pockwinse SM, Wilming LG, Conlon DM, Stein GS, Lian JB (1992): J Cell Biochem 49:310–323.

Rodan G, Heath JK, Yoon K, Noda M, Rodan SB (1988): "Cell and Molecular Biology of Vertebrate Hard Tissues." Ciba Found. Symp. 136:78–91.

Shapiro IM, Lee NH (1975): Clin Orthop 106:323–329.

Shapiro IM, Lee NH (1978): Metab Bone Dis Relat Res 1:173–177.

Shapiro IM, Golub EE, Kakuta S (1982): Science 217:950–952.

Stein GS, Lian JB, Owen TA (1990): FASEB J 4:3111–3123.

STIC-ILL 10509490 QH431.N363

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

37 2884

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24. 8841201
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic

Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murin embryos with a non-permissive genetic background

Jim McWhir[1], Angelika E. Schnieke[2], Ray Ansell[1], Helen Wallace[1], Alan Colman[2], Ann R. Scott[2] & Alexander J. Kind[2]

Embryonic stem (ES) cells enable the engineering of precise modifications to the mouse genome by gene targeting. Although there are reports of cultured cell contributions to chimaeras in golden hamster[1], rat[2] and pig[3], definitive ES cell lines which contribute to the germline have not been demonstrated in any species but mouse. Among mouse strains, genetic background strongly affects the efficiency of ES isolation, and almost all ES lines in use are derived from strain 129 (refs 1,4,5) or, less commonly, C57BL/6 (refs 6–8). The CBA strain is refractory to ES isolation and there are no published reports of CBA-derived ES lines. Hence, CBA mice may provide a convenient model of ES isolation in other species. In ES derivation it is critical that the primary explant be cultured for a sufficient time to allow multiplication of ES cell progenitors, yet without allowing extensive differentiation[9,10]. Thus, differences in ES derivation between mouse strains may reflect differences in the control of ES progenitor cells by other lineages within the embryo. Here we describe a strategy to continuously remove differentiated cells by drug selection, which generates germline competent ES lines from genotypes that are non-permissive in the absence of selection.

We were interested in determining whether ES progenitor cells analogous to those found in strain 129 were also present in explants of predominantly CBA embryos. If so, then the failure of these genotypes to give rise to ES lines may lie in the tighter regulation of ES progenitors by other lineages. The DNA construct, pOctneo1 was designed to selectively confer G418 resistance on undifferentiated ES cells and ES cell progenitors. It consists of 1.9 kb of sequences 5' to the *Oct3/4* coding region[11] linked to the bacterial *neo* gene. *Oct3/4* is a transcription factor expressed in undifferentiated epiblast, ES and EC cells, and is strongly down regulated following differentiation of EC cells *in vitro*[12] and of the epiblast *in vivo*[13].

Expression of pOctneo1 was first assessed in established ES cells. HM-1 ES cells[5] were transfected with either pOctneo1, a constitutive control plasmid pMTneo which expresses *neo* under the direction of the metallothionein promoter, or a reporter plasmid, pOctLacZ. Transient expression of β-galactosidase from pOctLacZ revealed low level expression from the 1.9-kb promoter, confirming previous findings[11]. Two pOctneo1 (ON2, ON4) and one pMTneo (MN6) stable clones were randomly selected and induced to differentiate by suspension culture in the absence of leukaemia inhibitory factor (LIF). In the absence of selection, each of the three clones undergoes extensive differentiation (Fig. 1*a, c, e*). Imposition of G418 selection does not affect the extent of differentiation of MN6 (Fig. 1*f*), but significantly reduces the number of differentiated cells present in ON2 and ON4 (Fig. 1*b, d*). These findings suggest that the 1.9-kb *Oct3/4* promoter is appropriately down-regulated following *in vitro* differentiation of ES cells, and are consistent with selective ablation of differentiated cells as they arise.

The effect of G418 selection on the efficiency of ES cell derivation was first examined in embryos which were permissive for ES isolation. This allowed transgenic lines capable of producing G418 resistant ES lines to be identified for backcrossing onto the non-permissive CBA background. Nine pOctneo1 transgenic lines were derived in F1 (C57BL/6 × CBA) mice. Embryos were produced by mating G1 hemizygous transgenic males to females of strain 129. Since both 129 and C57BL/6 strains can give rise to ES lines at high frequency, the genetic background of these embryos (25% CBA, 25% C57BL/6, 50% 129) was expected to be permissive for ES isolation. Embryos were apportioned to culture in medium with or without G418 in a ratio of approximately 2:1 to account for loss under selection of embryos lacking the *pOctneo1* transgene. G418 resistant ES cell lines were derived at heightened frequency from blastocyst stage embryos from five of the transgenic lines (Table 1*a*) using the standard ES cell derivation protocol[9,10].

These results encouraged us to test drug selection in embryos with a predominantly CBA, and hence non-permissive, genetic background. To confirm that our CBA stock was non-permissive, 63 100% CBA embryos were entered into culture for ES isolation. No lines were isolated and explants rapidly differentiated, showing little or no expansion of the inner cell mass (ICM) (Fig. 2*c, d*). G3 males homozygous for the *pOctneo1* transgene were then mated to CBA females to produce embryos of genetic background 87.5% CBA, 12.5% C57BL/6.

Non-permissive embryos (70) were explanted and ES cell derivation was carried out according to the 'standard protocol'[9]. In the absence of selection non-permissive explants differentiated extensively during the first 5 days in culture and showed no evidence of undifferentiated cells. Under G418 selection explants were extremely small after 8 days in culture. Although there was some indication that differentiation was less extensive than in the non-selected group, no explants from either group survived disaggregation when carried out on or before the 8th day in culture and no ES cell lines were established (Table 1*b*).

These observations led us to extend the period of culture prior to disaggregation to 14 days, and to reduce G418 concentration from 100 μg/ml to 50 μg/ml, whereupon all unselected explants showed extensive differentiation within 10 days (Fig. 2*e*). At the time of disaggregation there were no identifiable ICM outgrowths, and no ES lines were derived after passage. A high proportion (50 of 86) of the explants in the selected group died before day 14. Explants from transgenic lines 72–49 and 72–9, however, produced 13 outgrowths composed of predominantly undifferentiated cells, resembling large colonies of an established ES line (Fig. 2*f*). Eight of these gave rise to ES cell lines after disaggregation and grew to confluence as essentially pure ES cells.

[1]*Roslin Institute, Department of Molecular Biology, Roslin, Midlothian, EH259PS, UK*
[2]*PPL Therapeutics, Roslin, Midlothian, EH259PP, UK*

Correspondence should be addressed to J.M.

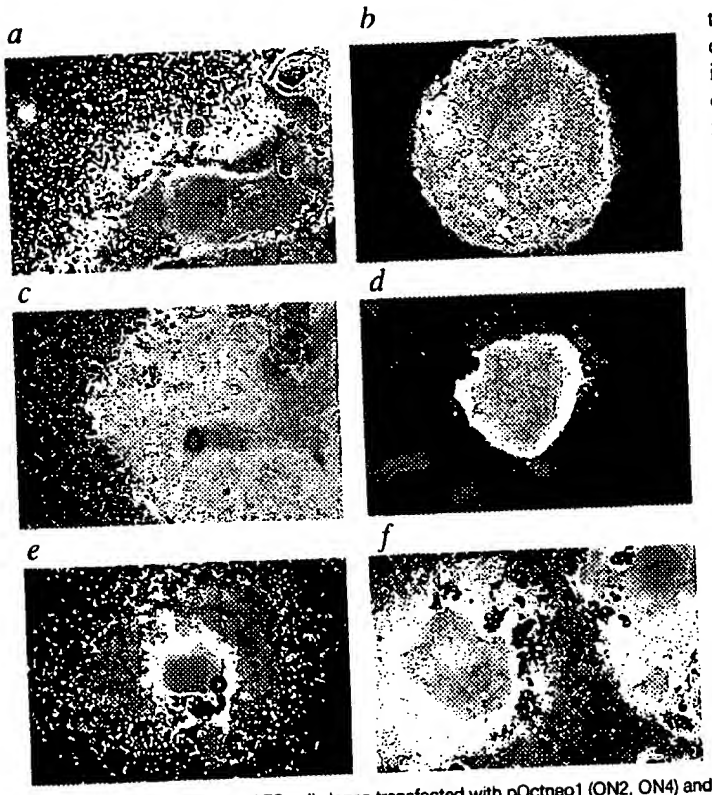


Fig. 1 *In vitro* differentiation of ES cell clones transfected with pOctneo1 (ON2, ON4) and pMtneo (MN6). The plates show embryoid bodies formed in suspension culture, then allowed to reattach to tissue culture plastic and cultured for 7 days. *a, c, e,* representative embryoid bodies formed in the absence of G418 by clones ON2, ON4 and MN6 respectively. *b, d, f* representative embryoid bodies formed in the presence of G418 by clones ON2, ON4 and MN6.

This result contrasts with conventional ES isolation in which primary outgrowths of the ICM contain several non-ES lineages[9]. Only upon further passage do small ES-like clones arise and this is always in conjunction with colonies of other lineages. Even when removed from the influence of other lineages many of these primary 'ES-like' colonies subsequently differentiate into trophectoderm[9]. Hence, the direct emergence of apparently pure ES cultures from the primary explant following drug selection is consistent with selective ablation of differentiated cells and represents an important simplification of the ES isolation procedure.

It may be significant that the 1.9-kb promoter fragment used provides only low levels of expression in ES cells[14]. Expression from the 1.9-kb promoter, while insufficiently powerful to provide strong staining with pOct-lacZ, nonetheless provides adequate levels of *neo* protein for G418 resistance. Low expression could lead to more efficient ablation of differentiated cells because it would reduce the number of G418 resistant differentiated cells arising from either leaky expression or persistance of protein following differentiation. This argument is consistent with the preferential establishment of ES lines from transgenic lines with low copy number (Table 1*c*).

Cytogenetic analysis of four lines revealed that all were of 40XY constitution and three of these lines gave rise to chimaeras following injection into MF1 (albino) blastocysts (Fig. 3*a*). One of two lines tested for germline transmission has been shown to contribute to the germ line at low frequency (Fig. 3*b*). Outbred strains such as MF1 generally produce lower frequencies of germline transmission than inbred strains, when used as host embryos[15–17], and we anticipate that this frequency will rise when chimaeras are made with inbred and hence less vigorous host strains. Even among inbred strains, specific combinations of ES line and host embryo strain are reported to show varying compatibility. Some C57BL/6 ES lines, for example, produce germline chimaeras in inbred BalbC embryos but not in embryos of the inbred strain C3H[6,7].

Similarly, chimaeras of 87.5% CBA ES lines with MF1 embryos produced in the present study failed to show areas of *agouti* colouration, apparently reflecting a poor contribution to follicle cells by CBA cells when in competition with MF1 cells. The ES line 39/1, however, gives rise to both *agouti* and non-*agouti*, (black) pigmented germline pups and is therefore heterozygous wild type at the *agouti* locus (Aa). Hence, failure to detect *agouti* colouration in chimaeras probably occurs due to a combination of low contribution by ES-derived cells to the follicle and reduced expression at the *agouti* locus due to heterozygosity. Pigmentation in the progeny of MF1 crosses with other strains is consistent with the MF1 genotype aa/BB/cc (ref. 18), and both black and *agouti* pups in the present study almost certainly arise from alleles contributed by ES line 39/1. The derivation of germline pups from 87.5% CBA ES cells, however, was confirmed by Southern blot (Fig. 3*c*), where we observed the presence of the pOctneo1 transgene with characteristic bands common to ES line 39/1 and to the transgenic line (72–9) used to generate that line.

The demonstration of ES cells from non-permissive embryos by drug selection supports the view that the genetic background of ES cell progenitors is not an autologous determinant of ES cell derivation, but that genetic background determines the level of suppression of ES proliferation. ES progenitor cells are present in predominantly CBA embryos, and it seems therefore that the significant difference between permissive and non-permissive mouse strains is the strength of the influence the embryo exerts over ES cell progenitors. Loss of embryonic control over ES precursors under selection

Table 1 ES cell derivation from pOctneo transgenic embryos

ES cell line derivation from permissive (25% CBA) embryos of five pOctneo1 transgenic mouse lines

a,	pOctneo1 transgenic lines (heterozygotes)						
	71–2	71–11	72–9	72–10	72–49	Total	Efficiency
–G418[a]	0/4	1/8	0/7	1/8	2/9	4/36	11.0%
+G418[a]	2/10	1/15	3/15	1/13	1/17	8/70	11.45% (22.8%[b])

ES cell line derivation from non-permissive (87.5% CBA) embryos of four pOctneo1 transgenic mouse lines

	pOctneo1 transgenic lines (homozygotes)					
b,	71–11	72–9	72–10	72–49	Total	Efficiency
Standard protocol						
–G418[a]	0/4	0/14	0/8	0/10	0/36	0%
+G418[a]		0/8		0/26	0/34	0%
c,						
14-day protocol						
–G418[a]	0/9	0/13	0/5	0/13	0/40	0%
+G418[a]	1/36	4/29	0/6	4/15	9/86	10.5%
Transgene copy number	>10	1–2	5–10	1–2		

Transgene copy number in each mouse line is shown in the bottom row.
[a]ES lines derived /blastocysts explanted into culture.
[b]Efficiency adjusted by a factor of 2 to allow for death of non-transgenic embryos.

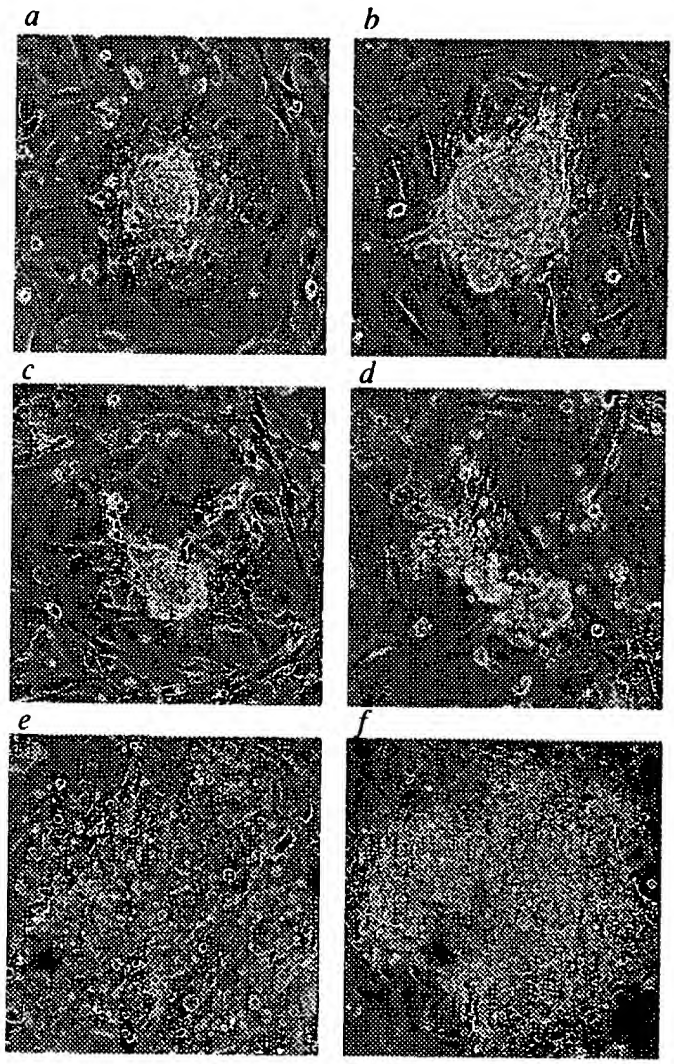


Fig. 2 *a*, Representative primary explants of permissive strain 129 embryos at 3 days in culture and, *b*, 5 days culture. Non-permissive CBA embryos at, *c*, 3 days culture and, *d*, 5 days culture. CBA pOctneo1 embryos at 14 days culture, *e*, without G418 selection and, *f*, with G418 selection.

probably results from the preferential ablation of non-ES lineages following down regulation of *neo* by the *Oct3/4* promoter, though this has not been conclusively demonstrated.

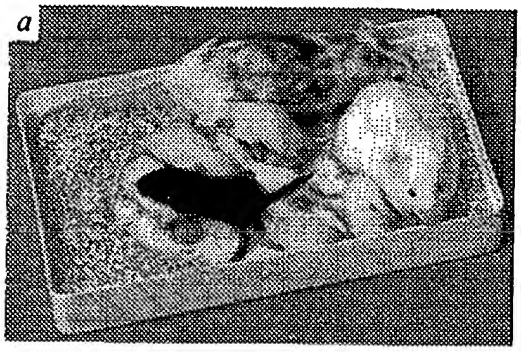
The approach taken in mice can be extended directly to small animals such as rat or rabbit where the generation of transgenic lines is not a problem, and possibly even to pigs, where fecundity and transgenic frequencies are relatively high. We have recently generated several transgenic lines of pigs carrying pOctneo1 and are currently evaluating the feasibility of porcine ES line derivation from transgenic embryos.

Methods

Clones and constructs. A 1,905-bp fragment consisting of 5' sequences flanking the *Oct3/4* gene and corresponding to positions −1876 to +29 in the sequence reported by Okazawa *et al.*[11] was amplified by PCR from strain 129 mouse genomic DNA. *Sal*I and *Hin*dIII sites were incorporated into the PCR primers to facilitate cloning. pOctneo1 was constructed by ligation of three fragments: 1) A *Sal*I–*Hin*dIII fragment containing the 1,905-bp Oct 3/4 promoter, 2) A 1.7-kb *Hin*dIII–*Eco*RI fragment containing a modified bacterial *neo* gene linked to 0.65 kb of the 3' end of the human growth hormone gene[19], 3) pUC8 linearised with *Eco*RI and *Sal*I. pMTneo was kindly provided by D. Melton and is described by Selfridge *et al.*[19]. pOctLacZ was constructed by insertion of a *Sal*I–HindIII fragment containing the 1,905-bp Oct3/4 promoter between *Sal*I and *Hin*dIII sites of the promoterless *LacZ* vector pON1 (ref. 20).

ES cell culture and transfection. Established ES cell lines were maintained without feeder layers in ES maintenance medium: Glasgow's Modified Eagle's Medium (GMEM; Life Technologies) supplemented with 400 U/ml recombinant murine leukaemia inhibitory factor (LIF; Life technologies), 0.1 mM MEM non-essential amino acids (Life Technologies), 5% newborn bovine serum, 5% fetal bovine serum and 0.1 mM β-mercaptoethanol (Sigma). Cell lines were cultured at 37 °C in a 5% CO_2 atmosphere in a humidified incubator.

Cells of the ES cell line HM-1 (ref. 5) were electroporated using standard conditions. ES cells, 10^7, were mixed with 100 μg linear DNA in 0.8 ml of HEPES-phosphate buffered saline (pH 7.5), given a single pulse of 800 V (path length 0.4 cm, 3 μF, Bio-Rad Gene Pulser) and plated at 10^6 cells per 10 cm dish. Stably transfected clones were isolated by selection with 300 μg/ml G418 (geneticin, Life Technologies) for 14 d.



b

pOctneo1 transgenic line	72–9		72–49	
87.5% CBA ES line	39/1	22/9	12/1	16/1
chimaeras/pups born	7/23	10/27	0/7	1/8
male chimaeras	4	6	0	1
germline chimaeras	1 (3/41)[a]			

[a] Germline pups expressed as a proportion of total pups born.

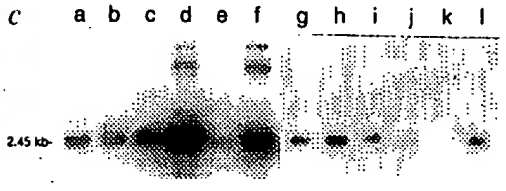


Fig. 3 *a*, A germline chimaera derived from line 39/1, its albino MF1 mate and a germline (*agouti*) and non-germline pup (albino) from the same litter. *b*, Chimaera formation and germline transmission of 87.5% CBA ES lines derived under G418 selection. *c*, Southern blot of *Bam*HI digested genomic DNA hybridized with a *neo* probe; From left to right, lanes *a–f*, 6 transgenic lines (71–19, 71–2, 71–10, 72–10, 72–9, 71–11); and lane *g*, ES line 39/1 derived from transgenic line 72–9; lanes *h–l*, germline pups GL1–GL5. Line 72–9 (lane *e*), in common with ES line 39/1 and the germline pups GL1, GL2, GL3 and GL5, shows a single characteristic 2.45-kb band. Germline pup GL4 (lane *k*) has not inherited the transgene.

In vitro differentiation of ES cells. Transfected ES cells were induced to differentiate both in the presence and absence of 500 μg/ml G418, by growth as aggregates in suspension culture, using a procedure similar to that described[9] for the formation of embryoid bodies. Transfected ES cell clones were grown to sub-confluence in tissue culture flasks, lightly trypsinised and clumps of cells transferred to 10 cm bacteriological petri dishes in differentiation medium (ES maintenance medium omitting LIF and newborn bovine serum). After 8 d in suspension, aggregates were induced to reattach by transfer to gelatinised TC dishes in ES maintenance medium. After a further 7 d culture cells were fixed for 10 min in 3:1 methanol:acetic acid and stained with crystal violet.

Transgenic mice and embryos. Transgenic mice were generated by pronuclear microinjection of pOctneo1 DNA into F1 (CBA X C57BL/6) embryos by standard methods[21]. The presence of the transgene was identified by Southern analysis of genomic DNA prepared from tail biopsies. Transgene copy numbers were estimated by comparing the signal intensity of the transgene in genomic DNA with that of diluted pOctneo1 plasmid DNA. G1 males were produced by mating transgenic founder animals to F1 (CBA X C57BL/6) mates. The G2 generation was produced by mating G1 males to CBA females. G2 animals of the same transgenic line were intercrossed and G3 males homozygous for the pOctneo1 transgene were identified by test mating and Southern analysis of fetal offspring.

Embryos with a genetic background derived from 50% 129, 25% CBA, 25% C57BL/6 were obtained by mating hemizygous pOctneo1 G1 males with strain 129 females. Embryos with a genetic background derived from 87.5% CBA, 12.5% C57BL/6 were obtained by mating homozygous pOctneo1 G3 males with CBA females.

ES cell derivation. Culture medium for derivation of ES lines (isolation medium) consisted of ES maintenance medium supplemented with fetal bovine serum to a final concentration of 15%. Where drug selection was applied, G418 was included at either 50 μg/ml or 100 μg/ml (selection medium). Newly derived ES cell lines were transferred from isolation medium to ES maintenance medium after passage four. Primary explants and new ES cell lines up to passage two or three were maintained on mitotically inactivated STO fibroblast feeder layers[9].

Manipulation of embryo explants for the derivation of ES cell lines was essentially as described[9]. Embryos were obtained from naturally mated females killed on day 3.5 p.c. Uterine horns were removed and flushed with approximately 0.5 ml of isolation medium. Embryos were washed three times in isolation medium and explanted in groups of 3–6 onto feeder layers in single wells of 24-well plates. At first passage, primary explants were disaggregated by gentle trypsinisation into clumps of cells, then transferred onto feeder layers in single wells of 24-well plates.

Culture of explants under selective and non-selective conditions was generally carried out in parallel, frequently using embryos from the same uteri. In an attempt to reduce possible operator bias, the person carrying out ES cell derivation from non-permissive embryos was not made aware of which explants were grown under selective or non-selective conditions. However, after ~10 days the behaviour of the cultures was clearly distinguishable.

Preparation of metaphase chromosomes and sexing. Metaphase chromosome spreads were prepared as described[9]. Sexing was determined by PCR, using primers to the Y-specific gene, SRY[22]; (5'–CCA TGT CAA GCG CCC CAT GAA TGC–3' and 5'–CAC TTT AGC CCT CCG ATG AGG CTG–3'). PCR conditions were: 45 s at 94 °C, 60 s at 65 °C, 60 s at 72 °C for 30 cycles plus 10 min at 72 °C. PCR products were analysed by agarose gel electrophoresis.

Generation and characterisation of chimaeras. CBA ES cells (87.5%) were introduced into strain MF1 blastocysts by microinjection and embryos transferred to pseudopregnant recipients using standard techniques[23]. Chimaeras were identified as mosaics of MF1 (albino) and CBA (pigmented). All chimaeras were test mated at sexual maturity to MF1 animals. Germline transmission from such matings could be identified by the appearance of black or agouti pups as 87.5% CBA ES lines are homozygous dominant at the *black* and *albino* loci respectively (BB/CC), and either heterozygous (Aa) or homozygous wild type (AA) at the *agouti* locus. Germline pups were also confirmed by Southern analysis of DNA prepared from tail biopsies.

Acknowledgements

We thank Y. Gibson and A. Scott for technical assistance. J.M., H.W. and R.A. are supported by the Biotechnology and Biological Sciences Research Council (U.K.). This work was partially funded by a grant (G9217459MB) from the Medical Research Council.

Received 6 May; accepted 30 July 1996.

1. Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morph.* 87, 27–45 (1985).
2. Iannaccone, P.M., Taborn, G.U., Garton, R.L., Caplice, M.D. & Brenin, D.R. Pluripotent embryonic stem cells from the rat are capable of producing chimaeras. *Dev. Biol.* 163, 288–292 (1994).
3. Wheeler, M.B. Development and validation of swine embryonic stem cells: a review. *Reprod. Fertil. Dev.* 6, 563–568 (1994).
4. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156 (1981).
5. Magin, T.M., McWhir, J. & Melton, D.W. A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. *Nucl. Acids Res.* 20, 3795–3796 (1992).
6. Kawase, E. *et al.* Strain difference in establishment of mouse embryonic stem (ES) cell lines. *Int. J. Dev. Biol.* 38, 385–390 (1994).
7. Lederman, B. & Burki, K. Establishment of a germ-line competent C57BL/6 embryonic stem cell line. *Exp. Cell. Res.* 197, 254–258 (1991).
8. Suemori, H. & Nakatsuji, N. Establishment of the embryo-derived (ES) cell lines from mouse blastocysts: effects of the feeder cell layer. *Dev. Growth Differ.* 29, 133–140 (1987).
9. Robertson, E.J. Embryo-derived stem cells. in *Teratocarcinomas and embryonic stem cells a practical approach.* (ed. Robertson, E.J.) 71–112 (IRL Press, Oxford, 1987).
10. Abbondanzo, S.J., Gadi, I. & Stewart, C.L. Derivation of embryonic stem cell lines. *Meth. Enzym.* 225, 803–823 (1993).
11. Okazawa, H.*et al.* The oct3 gene, a gene for an embryonic transcription factor, is controlled by a retinoic acid repressible enhancer. *EMBO J.* 10, 2997–3005 (1991).
12. Ben-Shushan, E., Pikarsky, E., Klar, A. & Bergman, Y. Extinction of *Oct-3/4* gene expression in embryonal carcinoma X fibroblast somatic cell hybrids is accompanied by changes in the methylation status, chromatin structure and transcriptional activity of the Oct-3/4 upstream region. *Mol. Cell Biol.* 13, 891–901 (1993).
13. Rosner, M.H. *et al.* A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* 345, 686–692 (1990).
14. Yeom, Y.I. *et al.* Germline regulatory element of *Oct-4* specific for the totipotent cycle of embryonal cells *Development* 122, 881–894 (1996).
15. Schwartzberg, P.L., Goff, S.P. & Robertson, E.J. Germ-line transmission of a *c-abl* mutation produced by targeted gene disruption in ES cells. *Science* 246, 799–803 (1989).
16 Dechiara, T.M., Efstratiadis, A & Robertson E. A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor 2 gene disrupted by targeting. *Nature* 345, 78–80 (1990).
17. Hooper, M.L. *Embryonal stem cells: introducing planned changes into the animal germline.* 147 (Harwood Academic Publishers, Switzerland, 1990).
18. Thompson, S., Clarke, A.R., Pow, A.M., Hooper, M.L & Melton, D.W. Germ line transmission and expression of a corrected *HPRT* gene produced by gene targeting in embryonic stem cells. *Cell* 56, 313–321 (1989).
19. Selfridge, J., Pow, A.M., McWhir, J., Magin, T.M. & Melton, D.W. Gene targeting using a mouse *HPRT* minigene/*HPRT*-deficient embryonic stem cell system: Inactivation of the mouse *ERCC-1* gene. *Somat. Cell Mol. Genet.* 18, 325–336 (1992).
20. Thomsen, D.R., Stenburg, R.M., Goins, W.F. & Stinski, M.F. Promoter regulatory region of the major immediate early gene of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA.* 81, 659–663 (1984).
21. Hogan, B., Costantini, F. & Lacey, E. *Manipulating the mouse embryo: A laboratory manual.* (Cold Spring Harbor Laboratory Press, New York. 1986).
22. Coward, P. *et al.* Polymorphism of a CAG tri nucleotide repeat within SRY correlates with B6.YDom sex reversal. *Nature Genet.* 6, 245–250 (1994).
23. Bradley, A. Production and analysis of chimaeras. in *Teratocarcinomas and embryonic stem cells a practical approach.* (ed. E.J. Robertson) 113–151 (IRL Press, Oxford, 1987).

JOURNAL OF MEDICINE

EVIDENCE FOR PROLIFERATION AND DIFFERENTIATION OF ENDOSTEAL CELLS INTO HEMOPOIETIC CELL LINES IN SHORT-TERM LIQUID CULTURE

Anwarul Islam*, Chester Glomski and Edward S. Henderson***

*Department of Medical Oncology,
Roswell Park Memorial Institute
666 Elm Street, Buffalo, NY 14263 and
**Department of Anatomical Sciences
School of Medicine, Faculty of Health Sciences
State University of New York at Buffalo
Buffalo, NY 14214

Key Words: Endosteal cells, Hemopoietic cells, Short-term liquid culture.

Abstract

To study the ability of endosteal cells to spontaneously differentiate *in vitro*, cells isolated from the endosteal bone surface (endosteal cells) were incubated in liquid suspension cultures containing fetal calf serum and antibiotics, but without chemical inducers of differentiation. Cultures were examined daily for the appearance of morphologically recognizable mature and differentiated hemopoietic cells. Culture outcomes were uniformly

Send correspondence to: Dr. Anwarul Islam, Department of Medical Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

consistent, showed an almost complete disappearance of the endosteal cells (which appeared as stimulated lymphoid and late stage normoblast-like cells once they are separated from the bone surfaces), and their replacement by differentiated and mature hemopoietic cells. The ability of endosteal cells to fully differentiate into mature hemopoietic cells *in vitro* indicates that endosteal cells represent the multipotential hemopoietic stem cells *in vivo*, and are equivalent of embryonal stage undifferentiated mesenchymal cells.

Introduction

The concept that endosteal cells (flattened endothelium-like cells that line the bony trabeculae) are true multipotential stem cells in the bone marrow, which are able to commit and differentiate into many different cell lines, including hemopoietic cells, is new (Islam, 1985). However, this concept is based primarily on morphologic observations made on fixed histologic sections. No direct evidence for this is available, though circumstantial and indirect evidence is now emerging (Islam, 1985; 1987). In recent years, culture techniques have been devised in which undifferentiated hemopoietic progenitor cells can be stimulated to give rise to differentiated and mature hemopoietic cells (Bradley and Metcalf, 1966; Palu *et al.*, 1979; Pluznik and Sach, 1965). These culture techniques have proven to be extremely useful in studying mouse and human hemopoiesis (Lind *et al.*, 1974).

In this study, endosteal cells from the femoral diaphyses of 8 to 10 week old C57BL/6J mice were investigated for their ability to proliferate and differentiate in liquid culture. The aims were to see if terminal differentiation into mature granulocytes and macrophages occurs, as it does in other *in vitro* culture systems, and to determine if differentiation into other hemopoietic cell lines was possible. If these were the results, this study would provide direct evidence to support our hypothesis that the endosteum contains primitive hemopoietic precursor cells that are able to proliferate and differentiate into hemopoietic pathways.

Materials and Methods

The 8 to 10 week old male C57BL/6J mice used in this study were obtained from West Seneca Laboratory (West Seneca, NY).

In order to obtain the endosteal cells, individual femur shafts from freshly killed animals were stripped of all adherent tissues by scrapping with a scalpel blade. Their epiphysial ends were clipped and one was placed into the distal end of a Pasteur pipette modified to hold a diameter similar to that of the femur. Loose and loosely adherent marrow-rich blood cells within the marrow cavity were expelled by repeated (8 to 12 times) vigorous flushing of the shaft with RPMI 1640 culture medium contained in a 35 mm plastic petri dish. The femurs were then split in half longitudinally and the remaining red marrow was washed away by shaking and waving the bones in the culture medium contained in the same petri dish. Then, split femurs were removed and placed into another similar size petri dish containing approximately 3 to 5 ml of tissue culture medium and the adherent endosteal cells were scrapped off with a pointed scalpel blade. The removed cells were passed through a Ficoll Hypaque gradient to remove cellular debris and to obtain an almost pure population of mononuclear endosteal cells. These cells were then washed twice in normal saline, resuspended, and the final volume was brought to 40 ml by adding RPMI 1640, supplemented with 20% fetal calf serum and antibiotics. Ten ml of this and the endosteal cell suspension was incubated in each of the four T-25 Corning plastic flasks. Cultures were maintained at 33° C in an atmosphere of 5% CO_2 in air, and were fed every three days by removing 75% of the medium and adding an equal volume of the fresh medium. Cultures were examined daily for two weeks and every two days cytocentrifuged preparations were made from the supernatants to observe the morphologic changes.

Results

The starting culture of endosteal cells consisted predominantly (60 to 70%) of undifferentiated, large- and medium-sized blast-like cells, with round nuclei, prominent nucleoli and a narrow rim of pale or deep blue, agranular cytoplasm (Figure 1) and a small population (30 to 40%) of lymphoid and late-stage normoblast-like cells (Figure 1). Occasionally maturing granulocytes (myelocytes and beyond) and mast cells were also seen.

Within 48 hours of culture, proliferation (as evidenced by cells in mitosis) and differentiation (as evidenced by the appearance of

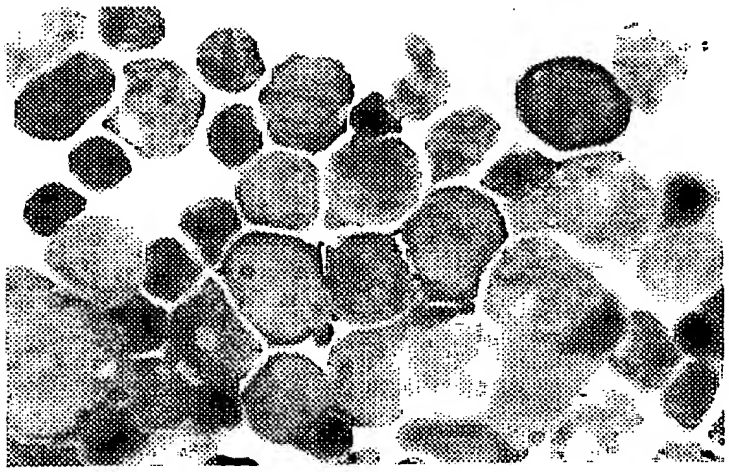


Figure 1.: Detailed morphology of endosteal cells obtained from the endosteal bone surfaces by means of the present isolation technique. Note the population of large and medium sized undifferentiated blast-like cells (majority), and a population (minority) of lymphoid and late-stage normoblast-like cells. Occasional maturing granulocytes can be seen. Wright-Giemsa stain. Original magnification x 1200.

Figure 2.: Polymorphonuclear maturation of endosteal cells in 8-day culture. Note almost complete disappearance of blast-like cells seen at day 0 of the culture. Wright-Giemsa stain. Original magnification x1200.

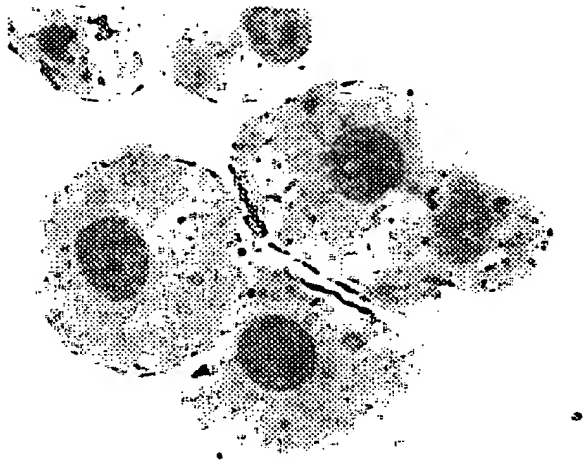


Figure 3.: Appearance of macrophages in 14-day culture. Wright-Giemsa stain. Original magnification x1200.

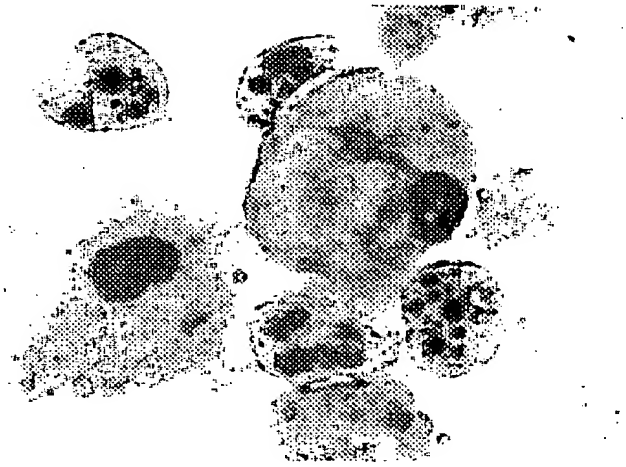


Figure 4.: A view from a 14-day culture showing granulocytes in the process of degeneration. Two macrophages can also be seen. Wright-Giemsa stain. Original magnification x1200.

maturing granuloid precursor cells) of endosteal cells began and, by day eight of culture, cells from the supernatant consisted almost entirely (over 90%) of mature granuloid precursor cells (Figure 2). Cells which had been almost uniformly undifferentiated and blast-like at the beginning of the culture period was reduced to less than 10% of the population. Although a small number of macrophages was seen on day four, they were not seen in moderate numbers until day ten. These cells were enlarged and filled with small vacules and granular material. The nuclei though still round, were somewhat smaller and the nucleoli were inconspicuous. By day 14, typical macrophages (Figure 3) were in the majority of cells and comprised almost 80% of the total nucleated cell population in the supernatant fluid. Mature and maturing granuloid precursors were reduced to less than 20% and a vast majority of them showed signs of degeneration (Figure 4). Mitotic activity had practically ceased, and the total number of cells in the cultures was greatly reduced. Undifferentiated blast-like cells were absent, though a variable number of small lymphoid and late stage normoblast-like cells were seen.

Discussion

Our previous observations (Islam, 1987; Islam *et al.*, 1980, 1984) indicated that cells firmly adherent to the endosteal bone surfaces are more primitive progenitor cells with a greater proliferative and differentiative potential. This led us to postulate that endosteal cells are the fixed totipotential stem cells in mammalian bone marrow and are the equivalent of embryonal level undifferentiated mesenchymal cells, which are capable of transformation and differentiation into osteoprogenitor cells (osteoblast and osteoclasts), stromal cells (fibroblast-like cells), and precursors of all hemopoietic cells in normal and stressed conditions under appropriate regulatory influence (Islam, 1985). In recent years, the development of *in vitro* culture techniques to assess the capability of progenitor cells to commit and differentiate into different hemopoietic cell lines (Bradley and Metcalf, 1966; Nakeff *et al.*, 1975; Pike and Robinson, 1970; Stephenson *et al.*, 1971) has proven extremely useful in the study of murine and human hemopoiesis. It is known that hemopoietic progenitor cells can be identified by their capacity to produce clones of differentiated progeny in culture (Bradley and

Metcalf, 1966; Pike and Robinson, 1970). If endosteal cells are indeed the ancestors of hemopoietic stem cells or if they do contain cells with hemopoietic potential, then they should also be able to produce hemopoietic differentiated progeny in such culture systems. To test our hypothesis, we attempted to use such a culture system to permit proliferation and differentiation of endosteal cells in the absence of any chemical inducers of differentiation. Although the endosteal cells in this culture system underwent spontaneous differentiation into granulocytes and macrophages, it is, however, not clear why they did not also differentiate or give rise to other types of hemopoietic cell, such as the erythroid or megakaryocytic cells. It is possible that the *in vitro* culture conditions used in this study some how favored the proliferation and differentiation of endosteal cells into granulocyte-macrophage pathways. Various chemical inducers of hemopoietic differentiation are now available (Donahue *et al.*, 1986; Sieff *et al.*, 1986; Sparrow and Williams, 1986) that can stimulate hemopoietic progenitor cells into commitment and differentiation into their various differentiated pathways. It would be interesting to see if such chemical inducers of differentiation could bring about the proliferation and differentiation of endosteal cells into cells other than granulocytes and macrophages.

It is generally accepted that morphologically recognizable bone marrow cells are derived from progenitor cells committed to a specific line of hemopoietic differentiation (Queesenbery and Levitt, 1979). The origin and morphologic identity of such progenitor cells is not yet known and the question whether there is a single pluripotent hemopoietic stem cell or a variety of stem cells each with a capacity of self-replication and maintenance is still unresolved (Loutit *et al.*, 1982). In early studies of the marrow in histologic sections, several authors have identified a generative hemopoietic zone in the subendosteal region and a maturational zone adjacent to the more centrally placed marrow sinusoids (Lennert, 1952; Weinbeck, 1938). High concentrations of stem cells have also been found near the bone surfaces (Gong, 1978), and a concentration gradient of such cells from the endosteum to the central axis of the femoral medullary cavity of mice has also been reported by Lord and Hendry, (1972) and Lord *et al.*,

(1975). In an autoradiographic study using tritiated thymidine as a pulse radiotracer for DNA synthesis, a kinetic gradient has also been demonstrated across the marrow section, with the highest proliferative rate in the subendosteal region (Shackney *et al.*, 1975). Although these observations pointed to some important functional activity at the endosteum, the endosteal cells have received very little attention and have never been considered a potential candidate for hemopoietic stem cells. In a recent report, we postulated that endosteal cells are the mutli-potential stem cells in the bone marrow which can give rise to various hemopoietic cells in normal and stressed conditions. In this report, we provide for the first time supportive evidence to our hypothesis and give direct morphologic evidence to suggest that endosteal cells do contain cells with hemopoietic potential, and are able to proliferate and differentiate into hemopoietic pathways.

References

Bradley, T.R. and Metcalf, D.: The growth of mouse bone marrow cells *in vitro*. Aust. J. Exp. Biol. Med. Sci. *44*: 287-300 (1966).

Donahue, R.E., Wang, E.A., Stone, D.K., Kamen, R., Wong, G.G., Sehgal, P.K., Nathan, D.G. and Clark, S.C.: Stimulation of haematopoiesis in primates by continuous infusion of recombinant human GM-CSF. Nature *321*: 872-875 (1986).

Gong, J.K.: Endosteal marrow: a rich source of hemopoietic stem cells. Science *199*: 1443-1445 (1978).

Islam, A.: Haemopoietic stem cell: a new concept: Leuk. Res. *9*: 1415-1432 (1985).

Islam, A.: Pattern of bone marrow regeneration following chemo-therapy for acute myeloid leukemia. J. Med. *18*: 108-122 (1987).

Islam, A., Catovsky, D. and Galton, D.A.G.: Histological study of bone marrow regeneration following chemotherapy for acute myeloid leukemia and chronic granulocytic leukaemic in blast transformation. Brit. J. Haematol. *45*: 535-540 (1980).

Islam, A., Catovsky, D., Goldman, J.M. and Galton, D.A.G.: Studies on cellular interactions between stromal and haemopoietic stem cells in normal and leukaemic bone marrows. Biblthca. Haematol. *50*: 17-30 (1984).

Lennert, K.: Zur Praxis der pathologisch-anatomischen Knochenmarksuntersuchung. Frankf. Z. Path. *63*: 267-299 (1952).

Lind, D.E., Bradley, M.L., Gunz, F.W. and Vincent, P.C.: The nonequivalence of mouse and human marrow culture in the assay of granulopoietic stimulating factors. J. Cell Physiol. *83*: 35-41 (1974).

Lord, B.I. and Hendry, J.H.: The distribution of haemopoietic colony-forming units in the mouse femur, and its modifications by X-rays. Brit. J. Radiol. *45*: 110-115 (1972).

Lord, B.I., Testa, N.G. and Hendry, J.H.: The relative spatial distribution of CFU-S and CFU-C in the normal mouse femur. Blood *46*: 65-72 (1975).

Loutit, J.F., Marshal, M.J., Nisbet, N.W. and Vaughan, J.: Versatile stem cells in bone marrow. Lancet *2*: 1090-1093 (1982).

Nakeff, A., Dicke, K.A. and Van Noord, M.J.: Megakaryocytes in agar cultures of mouse bone marrow. Ser. Haemat. *8*: 4-21 (1975). Author - check journal abbreviations.

Palu, G., Powles, R., Selby, P., Summersgill, B.M. and Alexander, P.: Patterns of maturation in short-term culture of human acute myeloid leukaemic cells. Brit. J. Cancer *40*: 719-730 (1979).

Pike, B.E. and Robinson, W.A.: Human bone marrow colony growth in agar gel. J. Cell Physiol. *76*: 77-84 (1970).

Pluznik, D.H. and Sachs, L.: The cloning of normal 'mast' cells in tissue culture. J. Cell. Comp. Physiol. *66*: 319-324 (1965).

Queesenbery, P. and Levitt, L.: Hemopoietic stem cells. N. Engl. J. Med. *301*: 755, 819, 868 (1979).

Shackney, S.E., Ford, S.S. and Witting, A.B.: Kinetic-micro-architectural correlations in the bone marrow of mouse. Cell Tissue Kinet. *8*: 505-516 (1975).

Sieff, C.A., Emerson, S.G., Mufson, A., Gesner, T.G. and Nathan, D.G.: Dependence of highly enriched human bone marrow progenitors on hemopoietic growth factors and their response to recombinant erythropoietin. J. Clin. Invest. *77*: 74-81 (1986).

Sparrow, R.L. and Williams, N.: Megakaryocyte colony stimulating factor: its identity to interleukin-3. Prog. Clin. Biol. Res. *215*: 123-128 (1986).

Stephenson, J.R., Axelrod, A.A., McLeod, P.C. and Shreeve, M.M.: Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro*. Proc. Natl. Acad. Sci. USA *68*: 1542-1546 (1971).

Weinbeck, J.: Die Granulopoese des kindlichen Knochenmarkes und ihre Reaktion auf Infectionen. Beitr. Path. Anat. *101*: 268-300 (1938).

STIC-ILL

5094904

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

372880

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

8907264

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic

Leukemia and Lymphoma, Vol. 21, pp. 17–23
Reprints available directly from the publisher
Photocopying permitted by license only

Gene Transfer-Mediated Generation of Drug-Resistant Hemopoiesis

FRANCESCO BERTOLINI,[1] CHIARA CORSINI,[1] LORENZA LAZZARI,[1] DAVIDE SOLIGO,[2] LUCIA DE MONTE,[3] MAUREEN WARD,[4] ARTHUR BANK[4] and GIROLAMO SIRCHIA[1]

[1]Centro Trasfusionale e di Immunologia dei Trapianti and [2]Centro Trapianti Midollo Osseo, Department of Hematology, Ospedale Maggiore Policlinico, Milano;[3] DIBIT, San Raffaele Scientific Institute, Milano, and [4]Department of Genetics and Development, Columbia University, New York, New York, U.S.A.

(Received 1 May 1995)

Autologous- or allogeneic-bone marrow transplantation are increasingly used to overcome the myelosuppressive effects of high dose chemotherapy administered to cancer patients. Transfer of the multidrug resistance (MDR) gene in hemopoietic progenitors has been proposed as a tool to administer higher and possibly more curative doses of chemotherapy. Murine models have demonstrated that retrovirus-mediated MDR transfer in bone marrow cells can render animals resistant to myeloablative doses of Taxol, and in vitro studies have shown that MDR-transduced human CD34+ cells can generate drug-resistant multipotential hemopoietic progenitors such as long term culture-initiating cells. Given these results, phase I clinical trials are currently under way to evaluate feasibility and treatment-related toxicity of MDR gene transfer in cancer patients by means of safe retroviral vectors. Finally, Taxol treatment of MDR transduced mice and human CD34+ cells have indicated that MDR is a dominant selectable marker in vitro and in vivo, and vectors carrying both MDR and non selectable genes such as β-globin or glucocerebrosidase could be used in the next future for gene therapy of inherited disorders like thalassemia or Gaucher disease.

KEY WORDS: Gene transfer generation of hemopoiesis drug resistant hemopoiesis

INTRODUCTION

Despite the use of appropriate doses of chemotherapy has led to major improvements in the treatment of neoplastic diseases, resistance to anticancer drugs frequently occurs. Resistance to multiple drugs was demonstrated about 25 years ago in epithelial neoplasms, and pioneering studies in this area indicated that if the tumor cells were resistant to one member of a family of drugs, the same cells were often resistant to the the other members of the family. In this context, Ling[1] proposed that a single efflux pump protein conferred resistance to a wide family of drugs by generating a reduction in the intracellular concentrations of these molecules. Subsequent laboratory work on neoplastic cells exhibiting drug resistance culminated in the isolation of the p170 glycoprotein (Pgp) efflux pump and of the multidrug resistance (MDR) family gene. The isolation of multiple forms of MDR cDNA in mouse and humans was possible, but led to the conclusion that only one group in each species is functionally active, i.e. MDR-1 in humans and MDR-3 in mouse.[2] Moreover, it was demonstrated that the activation of MDR confers resistance to chemotherapy agents such as colchicine, VP-16, vincristine, adriamycin, Taxol and others.[3] Since MDR-driven drug resistance appears to be a major clinical problem in many cancer patients, an intense research work has been devoted to the MDR gene family. In fact, from 1993 to 1994 more than 600 scientific papers regarding MDR gene expression and/or Pgp function have been referenced in *Index Medicus* and *Medline*. Although the physiological role of Pgp has not been fully elucidated, MDR transport function has been found to be independent of the physiological movement of ions, and a wide variety of compounds such as verapamil, cyclosporine and other chemosensitizers have been demonstrated to inhibit MDR

Address for correspondence: Francesco Bertolini, MD, PhD, Centro Trasfusionale e di Immunologia dei Trapianti, Department of Hematology, Ospedale Maggiore Policlinico, via Francesco Sforza 35, 20122 Milan Italy

transport in a subset of cancer patients.[4–5] This notwithstanding, significant side effects were found to hamper drug-mediated MDR inhibition in patients.[5] Despite impressive progress in the comprehension of MDR-related molecular biology and biochemistry,[6–7] cell machinery of MDR function, as revised by Simon and Schindler,[8] is still far from being fully understood. A novel, interesting approach with possible clinical applications has been recently proposed by Kobayashi *et al*,[9] who designed a hammerhead ribozyme able to decrease MDR RNA in MDR-expressing tumor cells. This tool may be applicable in treatment of cancer patients to reverse drug resistant tumors back to sensitivity.

RATIONALE FOR MDR PHENOTYPE TRANSFER

The treatment of choice for an increasing number of oncohematological diseases currently includes myelosuppressive doses of chemotherapy followed by autologous or allogeneic hemopoietic progenitor cell transplant.[10] A strategy able to make these progenitors and their offspring resistant to the toxic effect of chemotherapy would permit to safely administer larger and possibly more effective doses of chemotherapy to patients. With this aim in mind a number of investigators have evaluated the feasibility of drug resistance gene transfer in hemopoietic progenitors.[11–15] In addition to benefits for cancer patients related to administration of larger doses of chemotherapy, the transfer of drug resistance genes can be used as a selectable marker to enrich for progenitor cells that are cotransduced with a nonselectable gene both in vitro or in vivo. As an example, the generation of a vector able to transfer both the drug resistance gene and the nonselectable β-globin gene could be an effective mechanism for enriching cell populations able to express the product of the β-globin gene. This approach could be of help in designing vectors for gene therapy of inherited disorders such as thalassemia and Gaucher disease, as suggested by Aran *et al*[16] who recently described a retroviral bicistronic vector able to transfer a cassette including MDR and glucocerebrosidase genes under the control of a single promoter.

Virus-mediated methods for gene transfer are at present more popular than physical cDNA transfection such as electroporation or particle acceleration because viruses can achieve both high transfer efficiency and stable integration. Among viral vectors, retroviruses are the most widely used, and at the moment more than 85% of approved gene transfer clinical trials involve retroviruses.[17] Retrovirus mediated transduction in hemopoietic progenitors has been investigated both in vitro and in primates, and preliminary results indicate that stable integration and autonomous replication can be achieved. In spite of these developments, some drawbacks should be considered. First, gene transfer efficiency is far from being ideal, since it ranges from 1 to 70% of CFU-GM and 0.1 to 30% of long term-culture-initiating cells (LTC-IC), a subset of early, multipotential progenitors considered as possible "stem cell candidates".[18] Secondly, in vivo studies in primates and humans have been associated until now with a frustrating lack of foreign gene expression over time.[19] In fact, retroviruses can introduce genes into a single and active chromatin region in each cell and obtain in some cases long-term expression, but they can infect only mitotic cells, and transcripted foreign genes are often inactivated over time. In this context, it is of interest that the goal of drug-resistant hemopoiesis by means of MDR gene transfer does not strictly require lifespan or long-term expression of the foreign gene, since MDR mRNA generation and Pgp expression are only necessary to overcome one or few cycles of high dose chemotherapy.

Although this review predominantly focuses on procedures for MDR gene transfer, it should be mentioned that the cDNA of other genes known to confer resistance to different drugs has recently been isolated and transfered in vitro into hemopoietic progenitors. Flasshove *et al*[13] have transfered a mutated dihydrofolate reductase cDNA into CD34+ cells in order to confer methotrexate (MTX) resistance. It should be noted that this transduction protocol permitted both selection and cytokine-mediated ex vivo expansion of transduced progenitors. Magni *et al*[14] and Webb and Sorrentino[15] have recently generated retroviral vectors capable of transfering the aldehyde dehydrogenase gene (Aldh). The product of this gene catalyzes the intracellular conversion of metabolites of alkylating drugs such as cyclophosphamide and maphosphamide to an inert compound. Aldh-transduced murine and human hemopoietic cell lines were found to survive in the presence of 10–25 μM maphosphamide, and these data demonstrated a direct relationship between Aldh expression and resistance to this alkylating agent. Interestingly, maphosphamide resistance could be reversed in vitro by an inhibitor of cytosolic Aldh.[14] Moreover, Kruh *et al*,[20] have established using the NIH/3T3 cell line that the transfer of mrp gene, a member of the ABC cassette superfamily of transporters including MDR gene, is capable of conferring a multidrug resistance phenotype different from Pgp expression. Further studies are currently ongoing to evaluate whether transfer of this novel gene can generate drug-resistant hemopoiesis.

IN VIVO MODELS OF MDR GENE TRANSFER

In 1989, Galski *et al*[21] transfered the MDR cDNA into the transcription unit of an expression vector, and introduced it into the blastocyst of a mouse embryo. Bone marrow progenitors of the resulting mice, but not other tissues, were modified by the MDR plasmid, so that the exogenous MDR gene and Pgp phenotype were expressed at all stages of hemopoietic maturation. These mice were studied for about two years, and both hemopoietic proliferation and differentiation were found to be normal. In the early 1990's, a retroviral vector for human MDR gene transfer was generated,[22] and in 1992 two groups autonomously described mouse models of MDR gene transfer in vivo.[22-23] After demonstration that a retroviral construct containing MDR cDNA was able to transfect the ecotropic NIH/3T3 retroviral packaging line GP+E86, colchicine-resistant producer clones were cocultured in the presence of different cytokine cocktails with bone marrow cells of mice previously treated with the antimetabolite 5FU. This strategy was chosen to induce stem cell cycling, and was associated to stable integration of MDR gene in mice hemopoietic progenitors. When transduced cells were transplanted into irradiated mice, MDR gene expression was detected in both bone marrow and mature peripheral blood cells for several months after transplant. Moreover, transduced cells were resistant to doses of Taxol in the 7–14 mg/Kg range. To test whether MDR gene can function as a dominant selectable marker in vivo, transduced mice received Taxol 6 months after transplant. On the second rechallenge with Taxol, MDR transduced mice showed a significant increase of the proportion of circulating leukocytes containing MDR provirus. In contrast, control animals transduced with the neo gene showed no change in the number of circulating marked cells after drug treatment. Interestingly, by means of tissue distribution analysis of MDR transduced cells it was possible to detect MDR provirus in both myeloid and lymphoid cells, indicating that multilineage cells were transduced and the marker was still present in their progeny. More recently, a third group proposed a stromal layer-mediated MDR transduction protocol,[24] and serially transplanted MDR-transduced bone marrow cells into each of six successive cohorts of mice treated by Taxol in order to allow for in vivo selection of transduced progenitors. The success of this approach further confirms that very early progenitors with self-renewal potential were among the transduced target cells.

MDR GENE TRANSFER IN HUMAN HEMOPOIETIC PROGENITORS

The availability of retroviral vectors of MDR transfer has led to steps toward optimization of MDR transfer in human hemopoietic progenitors. First, Ward *et al*[11] described successful MDR transduction in human bone marrow-derived progenitors. By means of two rounds of exposure to MDR retroviral supernatant, these authors were able to transduce both low density and purified CD34+ cells, but transfer efficiency was significantly higher in purified CD34+ cells. Soft gel assays showed that after exposure to MDR supernatants, 18–70% of BFU-E and 30–60% of CFU-GM contained the transferred gene. Up to 11% of the progeny of these cells expressed increased amounts of Pgp, and flow cytometry studies demonstrated that transduced cells were enriched in Pgp-expressing cells after exposure to Taxol. Moreover, the same paper described the first evidence that not only committed CFU but also LTC-IC can be transduced by means of MDR retroviral vectors. Using the same retroviral vector developed by Ward *et al*,[11] other investigators[12,25-26] reported the utilization of cord blood, bone marrow and peripheral blood low density and purified CD34+ cells as targets for MDR gene transfer. In these studies, cells were cocultivated for 48 hours with the irradiated MDR retroviral producer line. Since MDR gene expression has been previously reported to occur at low levels in both hematopoietic progenitors and in peripheral blood cells, efficiency of MDR gene transfer was assessed by four different assays. First, transduced cells were cultured in the presence of SCF and IL-3 and selected by the presence of doxorubicin, colchicine and/or Taxol. In uninfected control, 1–2% of CFU-GM, CFU-GEMM and LTC-IC were found to be drug-resistant, while 11–33% of original clonogenic and LTC-IC activity was found after 2 weeks of culture of transduced cells. In these liquid culture conditions, efficiency of MDR transfer was significantly enhanced by prestimulation with cytokines, and transfer efficiency in LTC-IC found to be significantly superior in cord blood-derived compared to bone marrow- and peripheral blood-derived progenitors ($p < 0.05$). Second, MDR gene expression was evaluated through polymerase chain reaction. MDR mRNA expression was very low or absent in cultures of uninfected controls, whereas, after drug selection, MDR mRNA levels in transduced cells was as high as in the MDR retroviral producer line evaluated as a positive control. Third, flow cytometric analysis of CD34 and Pgp expression indicated that after MDR transduction and two weeks of culture/drug selection, membrane expression of Pgp was found on 17–25% of viable CD34+ cells versus 2–3% of untransduced controls. Furthermore by means of APAAP staining, transduced and cultured cells were found to express Pgp on plasma and nuclei membrane, while no specific localization was found in untransduced controls. Taken together, these data indicate that drug-resistant hemopoiesis could be generated by MDR gene transfer in human hemopoietic progenitors from bone marrow, peripheral

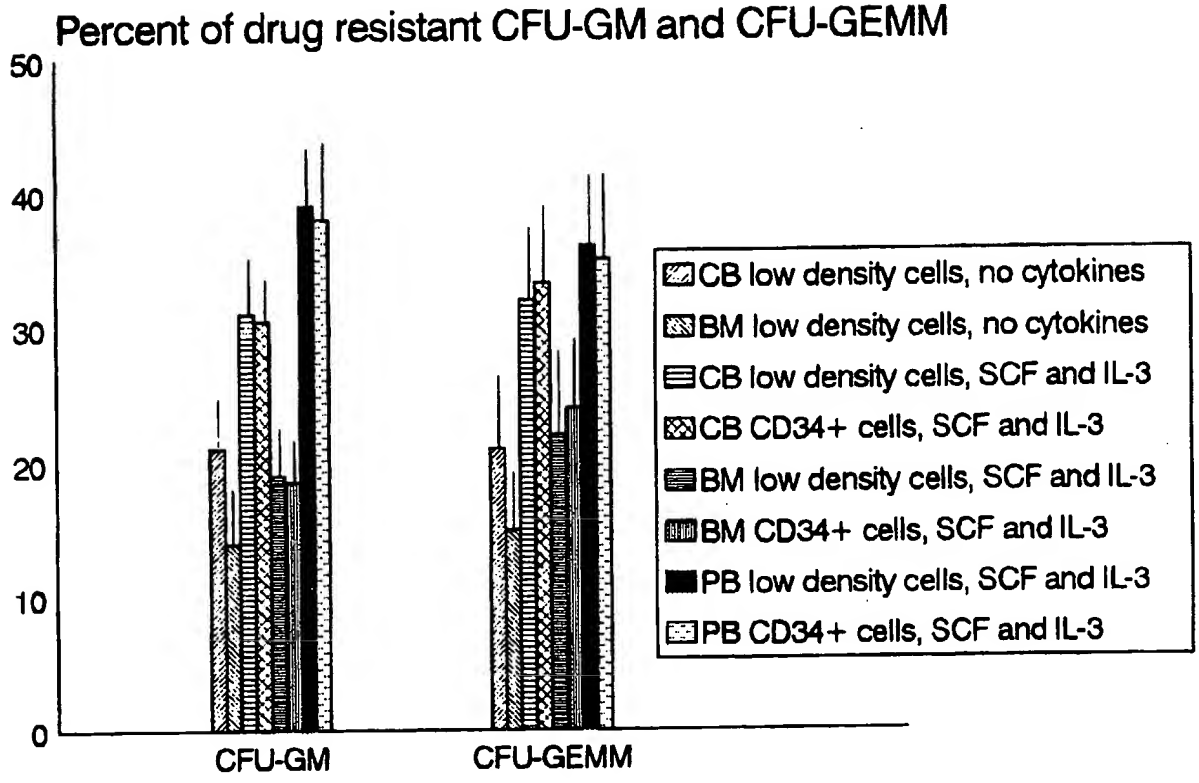


Figure 1 Infection efficiency of cord blood (CB), bone marrow (BM) and peripheral blood (PB) low density and CD34+ cells cocultivated in the presence of the A12M1 producer cell line developed by Ward *et al*[11] in order to transfer the MDR gene. PB-derived progenitors were collected by apheresis after *sc* administration of 10μg/Kg G-CSF for 5 days to healthy volunteers. On 5 occasions, low density and purified CD34+ CB, BM and PB cells were cultured for 24–48 hours in presence of SCF and IL-3 before transduction, and in 5 studies this stimulation procedure was not done. After MDR gene transfer, cells were cultured in presence of doxorubicin, colchicine or taxol, and uninfected controls were also evaluated. Results are expressed as mean ± 1 SD of the percentage of CFU-GM and CFU-GEMM found to be drug-resistant after transduction and drug selection. When cells were primed by citokines before transduction, the percentage of CFU found to be drug-resistant was superior to that observed after MDR transfer without prestimulation with cytokines ($p < 0.01$). MDR transfer efficiency was superior in PB cells compared to CB and BM cells, and in CB cells compared to BM cells ($p < 0.01$). In uninfected control ($n = 5$), 1–2% of CFU-GM and CFU-GEMM were found to be drug resistant (data not shown).

blood or cord blood. At the present time, two hypothesis could explain the higher retrovirus-mediated gene transfer efficiency observed in cord blood compared to bone marrow progenitors. Although the number of mitotic cells seems to be similar in cord blood and in bone marrow, Traycoff *et al*[27] have recently demonstrated that in cord blood progenitors the exit from G0/G1 phases of cell cycle is more rapid than in bone marrow progenitors. Moreover, an autocrine loop production of cytokines such as IL-3 and GM-CSF has been recently demonstrated in cord blood but not in bone marrow progenitors.[28] Strategies for further improvement of MDR gene transfer efficiency are currently under investigation, and involve optimization of cytokine combinations to stimulate exit of progenitors from G0/G1 cell cycle, the use of a stromal cell layer to generate niches in which LTC-IC could enter in S cell cycle phase, and continuous flow culture conditions. All these procedures could be of help in mimicking the physiologic process of blood cell generation and favor MDR gene transfer.

CLINICAL TRIALS INVOLVING MDR GENE TRANSFER

At the present time, three different proposals of clinical protocols including MDR gene transfer into human hemopoietic progenitors have been accepted. At Columbia University New York, USA and at the National Cancer Institute Bethesda, USA, breast, ovarian and brain cancer patients will undergo high dose chemotherapy and receive autologous CD34+ progenitors from peripheral blood and

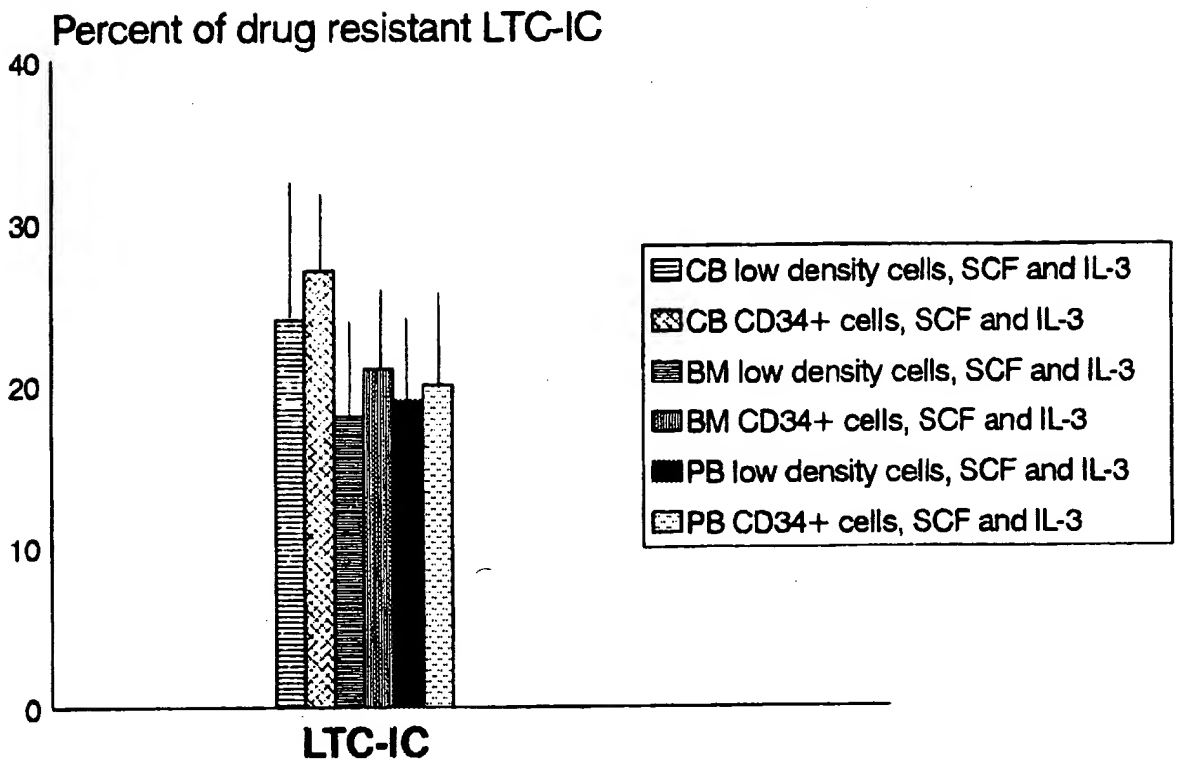


Figure 2 Infection efficiency in long term culture-initiating cells (LTC-IC) derived from cord blood (CB), bone marrow (BM) and peripheral blood (PB). PB-derived progenitors were collected by apheresis after *sc* administration of 10μg/Kg G-CSF for 5 days to healthy volunteers. Low density and CD34+ cells were cocultivated in the presence of the A12M1 producer cell line developed by Ward *et al.*,[11] and LTC-IC were evaluated following the method proposed by Sutherland and Eaves.[18] On 3 occasions, low density and purified CD34+ CB, BM and PB cells were cultured for 24–48 hours in presence of SCF and IL-3 before transduction. After MDR gene transfer, cells were cultured in presence of doxorubicin, colchicine or taxol, and uninfected controls were also evaluated. Results are expressed as mean ± 1 SD of the percentage of LTC-IC found to be drug-resistant after transduction and drug selection. Transfer efficiency was superior in CB cells compared to PB and BM cells ($p < 0.05$). In uninfected control ($n = 3$), 1–6% of CFU-GM and CFU-GEMM were found to be drug resistant (data not shown).

bone marrow, 70% unmanipulated and 30% previously transduced with a retroviral vector expressing MDR cDNA.[29-30] Marrow biopsies will be evaluated for the presence and expression of the MDR gene. These studies will investigate the safety of the transduction procedure in patients, and measure the transfer and expression of the MDR gene at the baseline and over different time periods. In one trial, patients with residual or progressive disease after autologous BMT will be treated with Taxol or vinblastine, asking the question whether chemotherapy amplifies the proportion of progenitors containing the MDR provirus. Moreover, sites of relapsed tumor will be biopsied to test for the presence of the MDR provirus. In a third clinical trial at the M.D. Anderson Cancer Center in Houston, Texas USA, after high dose chemotherapy, ovarian cancer patients will receive autologous CD34+ bone marrow cells, 50% unmanipulated and 50% transduced with a MDR retroviral vector.[31] After marrow rescue, every three weeks patients will receive Taxol at increasing doses, in order to promote in vivo selection of progenitor cells which have retained the MDR transgenome. If safety will be demonstrated in these pilot studies, subsequent phase II trials will be designed to assess efficacy of MDR gene transfer in reducing hematologic toxicity from high dose chemotherapy. In the autologous transplantation setting, a key-factor is the availability of purified hemopoietic progenitors free of contaminating neoplastic cells, which are at risks of being transduced with the MDR gene. Although methods for CD34+ progenitor cell purification are currently available, at the present time they achieve a 70–90% CD34+ cell purity,[29-31] and exclusion of neoplastic cell contamination by immunohistochemistry, flow cytometry or molecular biology is still required in all clinical trials currently under way involving transduction of CD34+ cells. Moreover, cancer patients will be enrolled in these studies on the basis

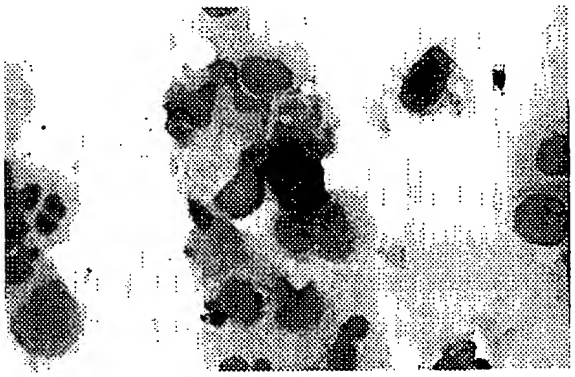


Figure 3 Transduced and drug-selected cord blood cells incubated with C219 monoclonal antibody reacting with Pgp. Immunoreactivity was revealed by means of APAAP and hematoxylin counterstaining. The majority of unstained cells display the typical morphology of well differentiated myeloid cells, while a single, small sized and intensively positive cell has staining localized both on the nucleus and in the cytoplasm. (See Color Plate I at the back of this issue.)

of the absence of CD34 antigen on tumor cells. In addition, the possibility of regenerating replication-competent retroviruses will be taken into acount, and retroviral vectors will undergo stringent safety testing to exclude wild-type retrovirus contamination.

CONCLUSIONS

Established mouse models and in vitro studies in human CD34+ progenitors indicate that MDR gene transfer is a feasible and attractive approach to generate drug-resistant hemopoiesis. This procedure could permit dose escalation of chemotherapy in cancer patients, and phase I clinical trials to evaluate treatment-related toxicity are in progress. In the meantime, studies in animal models will indicate whether transfer of other drug resistance genes such as dihydrofolate reductase, aldh and mrp could be a feasible approach to further protect hemopoiesis during chemotherapy. Finally, investigators are developing vectors carrying both MDR and nonselectable genes such as β-globin. These vectors could be of most interest for gene therapy of hematological inherited diseases, and used following a two step-approach. In vitro, progenitors could be transduced with MDR and the curative gene, purified by means of drug selection and expanded ex vivo by liquid culture in the presence of cytokines. Subsequently, patients could receive progenitors transduced with MDR and the curative gene and undergo repeated administration of Taxol to gradually select transduced progenitors without the need of myeloablation.

REFERENCES

1. Ling, V. (1989) Does P-glycoprotein predict response to chemotherapy? (Editorial). *JNCI*, **81**, 84–85.
2. Scotto, K. W. and Bertino, J. R. (1994) Chemotherapy susceptibility and resistance. In *The Molecular Basis of Cancer*, edited by J. Mendelsohn, P. M. Howley, M. A. Israel and L. A. Liotta, pp. 387–400. Philadelphia: W. B. Saunders.
3. Childs, S. and Ling, V. (1994) The MDR superfamily of genes and its biological implications. In *Important Advances in Oncology*, edited by V. T. De Vita, jr., S. Hellman and S. R. Rosemberg, pp. 21–36. Philadelphia: J. B. Lippincott.
4. Weaver, J. L., Szabo, G. jr., Pine, P. S., Gottesmann, M. M., Goldenberg, S., and Aszalos, A. (1993) The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multidrug transporter. *Int. J. Cancer*, **54**, 456–461.
5. Raderer, M. and Scheithauer, W. (1993) Clinical trials of agents that reverse multidrug resistance, a literature review. *Cancer*, **72**, 3553–3563.
6. Gottesmann, M. M. and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.*, **62**, 385–427.
7. Endicott, J. A. and Ling, V. (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann. Rev. Biochem.*, **58**, 131–171.
8. Simon, S. M. and Schindler, M. (1994) Cell biological mechanisms of multidrug resistance in tumors. *Proc. Natl. Acad. Sci. USA*, **91**, 3497–3504.
9. Kobayashi, H., Dorai, T., Holland, J. F., and Ohnuma, T. (1994) Reversal of drug sensitivity in multidrug-resistant tumor cells by an MDR1 (PGY1) ribozyme. *Cancer Res.*, **54**, 1271–1275.
10. Forman, S. J., Blume, K. G., and Thomas, E. D. (Editors, 1994) Bone marrow transplantation. Boston: Blackwell Scientific Publications.
11. Ward, M., Richardson, C., Pioli, P., Smith, L., Podda, S., Goff, S., Hesdorffer, C., and Bank, A. (1994) Transfer and expression of the human multiple drug resistance gene in Human CD34+ cells. *Blood*, **84**, 1408–1414.
12. Bertolini, F., De Monte, L., Corsini, C., Lazzari, L., Lauri, E., Soligo, D., Ward, M., Bank, A., and Malavasi, F. (1994) Retrovirus-mediated transfer of the multidrug resistance gene into human haematopoietic progenitors. *Br. J. Haematol.*, **88**, 318–324.
13. Flasshove, M., Banerjee, D., Mineishi, S., Li, M., Bertino, J. R., and Moore, M. A. S. (1995) Ex vivo expansion and selection of human CD34+ peripheral blood progenitor cells after introduction of a mutated dihydrofolate reductase cDNA via retroviral gene transfer. *Blood*, **85**, 566–574.
14. Magni, M., Shammah, S., Schirò, R., Bregni, M., Siena, S., Di Nicola, N., Dalla-Favera, R., and Gianni, A. M. (1994) Gene therapy for drug-induced myelotoxicity: Induction of cyclophosphamide resistance by aldheyde dehydrogenase-1 gene transfer (abstract). *Blood*, **84** (suppl 1), 357a.
15. Webb, M. and Sorrentino, B. P. (1994) Retroviral transfer of the human cytosolic aldheyde dehydrogenase gene as a means to generate cellular cyclophosphamide resistance. *Blood*, **84** (suppl 1), 357a.
16. Aran, J. M., Gottesman, M. M., and Pastan, I. (1994) Drug-selected coexpression of human glucocerebrosidase and P-glycoprotein using a bicistronic vector. *Proc. Natl. Acad. Sci. USA*, **91**, 3176–3180.
17. Hodgson, C. P. (1995) The vector void in gene therapy. *Bio/Technology*, **13**, 222–225.
18. Sutherland, H. J. and Eaves, C. J. (1994) Long-term culture of human myeloid cells. In *Culture of hematopoietic cells*, edited by R. I. Freshney, I. B. Pragnell and M. G. Freshney, pp. 139–162. New York: Wiley-Liss.
19. Bodine, D. M., Seidel, N. E., Gale, M. S., Nienhius, A. W., and Orlic, D. (1994) Efficient retrovirus transduction of mouse pluripotent hematopoietic stem cells mobilized into the peripheral blood by

treatment with granulocyte colony-stimulating factor and stem cell factor. *Blood*, **84**, 1482–1491.
20. Kruh, G. D., Chan, A., Myers, K., Gaughan, K., Miki, T., and Aaronson, S. A. (1994) Expression complementary DNA library transfer establishes mrp as a multidrug resistance gene. *Cancer Res.*, **54**, 1649–1652.
21. Galski, H., Sullivan, M., Willingham, M. C., Chin, K. W., Gottesman, M. M., and Pastan, I. (1989) Expression of a human multidrug resistance cDNA (MDR1) in the bone marrow of transgenic mice: resistance to daunomycin-induced cytopenia. *Mol. Cell. Biol.*, **9**, 4357–4363.
22. Podda, S., Ward, M., Himelstein, A., Richardson, C., Flor-Weiss E. de la, Smith, L., Gottesman, M., Pastan, I., and Bank, A. (1992) Transfer and expression of the human multiple drug resistance gene into live mice. *Proc. Nat. Acad. Sci. USA*, **89**, 9676–9680.
23. Sorrentino, B. P., Brandt, S. J., Bodine, D., Gottesman, M., Pastan, I., Cline, A., and Nienhius, A. W. (1992) Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of Human *MDR1*. *Science*, **257**, 99–103.
24. Hanania, E. G. and Deisseroth, A. B. (1994) Serial transplantation shows that early hematopoietic precursor cells are transduced by MDR-1 retroviral vector in a mouse gene therapy model. *Cancer Gene Therapy*, **1**, 21–26.
25. Bertolini, F., De Monte, L., Corsini, C., Lazzari, L., Lauri, E., Soligo, D., Malavasi, F., Ward, M., Bank, A., and Sirchia, G. (1994) Transfer of the the multidrug resistance gene into human hematopoietic progenitors collected from bone marrow, peripheral blood and cord blood. *Blood*, **84** (suppl 1), 356a.
26. Ward, M., Richardson, C., Pioli, P., Avello, J., Reiss, R., Hesdorffer, C., and Bank, A. (1994) MDR gene transfer and expression into peripheral blood progenitor cells. *Blood*, **84** (suppl 1), 356a.
27. Traycoff, C. M., Abboud, M. R., Laver, J., Clapp, D. W., and Srour, E. F. (1994) Rapid exit from G0/G1 phases of cell cycle in response to stem cell factor confers on umbilical cord blood CD34+ cells an enhanced ex vivo expansion potential. *Exp. Hematol.*, **22**, 1264–1272.
28. Schibler, K. R., Li, Y., Ohls, R. K., Nye, N. C., Durham, M. C., White, W., Liechty, K. W., Le, T., and Christensen, R. D. (1994) Possible mechanisms accounting for the growth factor independence of hematopoietic progenitors from umbilical cord blood. *Blood*, **84**, 3679–3684.
29. Clinical protocol: Human MDR gene transfer in patients with advanced cancer. *Human Gene Therapy*, **5**, 1151–1160.
30. Clinical protocol: Retroviral mediated transfer of the human MDR1 gene into hematopoietic stem cells during autologous transplantation after invasive chemotherapy for metastatic breast cancer. *Human Gene Therapy*, **5**, 891–911.
31. Clinical protocol: Use of safety-modified retroviruses to introduce chemotherapy resistance sequences into normal hemetopoietic cells for chemoprotection during the therapy of ovarian cancer: a pilot trial. *Human Gene Therapy*, **5**, 1507–1522.

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic

ANTICANCER RESEARCH *15*: 1873-1882 (1995)

Development of Human Leukemia U-937 Cell Sublines Resistant to Doxorubicin: Induction of Differentiation and Altered Sensitivities to Topoisomerase-Directed Drugs

P. PANTAZIS[1], A. DEJESUS[1], J. EARLY[1], R. RODRIGUEZ[1], D. CHATTERJEE[2], Z. HAN[2], J. WYCHE[2] and B. GIOVANELLA[1]

[1]*The Stehlin Foundation for Cancer Research at St. Joseph Hospital, Houston, TX;*
[2]*Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI, U.S.A.*

Abstract. *Cell sublines resistant to doxorubicin (DOX) were developed from the human leukemia cell line, U-937/WT, exposed to stepwise DOX increases. In contrast to U-937/WT cells, the DOX-resistant U-937/RD cells have longer doubling time; are more differentiated along the monocytic lineage as determined by the presence of morphological features and mRNA coding for the monocyte colony-stimulating factor-1 receptor; synthesize the apoptosis-associated Bax protein; are less sensitive to apoptosis-inducing topoisomerase II-directed drugs, apparently because of increased synthesis of P-glycoprotein; and are practically non-tumorigenic when xenografted in nude mice. However, U-937/WT and U-937/RD cells exhibit similar sensitivity to the apoptosis-inducing drug 9-nitrocamptothecin. These findings suggest that several mechanisms are involved in the development of DOX-resistance in U-937 cells, and further, 9-nitrocamptothecin can overcome resistance to DOX. These findings may have clinical implications.*

The antitumor drug doxorubicin (DOX) is a naturally produced antibiotic frequently prescribed in therapy of a broad spectrum of human cancers including leukemias, lymphomas, and solid tumors (1,2). The antineoplastic or antiproliferative action of DOX is reportedly generated by at least seven different means (3), but none of these means has been singled out as the most important in inducing clinical response in a particular case. Like several other antibiotics, DOX intercalates between base pairs of DNA (4) and generates toxic oxygen-free radicals, which cause DNA breaks followed by inhibition of DNA-directed RNA synthesis, protein synthesis, glutathione synthesis, defective mitoses, and high rates of mutation (5-8). Intercalation of DOX into chromatin DNA results in topological changes in the region of intercalation, which in turn interfere with the breakage-reunion reaction of the mammalian DNA enzyme topoisomerase II (9). Similarly, DOX inhibits topoisomerase I-induced cleavage of DNA in a cell-free system (10), whereas a tumor cell line with decreased synthesis of topoisomerase I was resistant to doxorubicin (11). Taken together, these results show that DOX indirectly inhibits cellular topoisomerase I and II activities in cultured cells and cell-free systems (10,12,13). Finally, continuous exposure of certain tumor cells to sublethal DOX concentrations may result in differentiation of these cells (13).

Despite the successful use of DOX in cancer chemotherapy, there are tumors that are either inherently insensitive, *i.e.*, intrinsically resistant, or acquire resistance to DOX after prolonged treatment of cancer patients with these drugs (2). Development of resistance to DOX in human leukemia U-937 cells has been associated with increased synthesis of Bcl-X_L (15), that is, the protein thought to function as a negative regulator (*i.e.*, suppressor) of the process of programmed cell death (apoptosis; 16). The effect of Bcl-X_L can be neutralized by the cellular protein Bax that is considered to function as an accelerator of apoptosis (17,18). However, resistance to DOX by most cancer cells is reportedly associated with increased synthesis of P-glycoprotein that confers multidrug resistance, MDR (19-21), increased synthesis of the multidrug resistance-associated protein, MRP (22,23), increased synthesis of the glucose-regulated stress protein, GRP78 (24), or a combination of these events (25,26). Increases in MRP and P-glycoprotein synthesis are associated with resistance to low and high DOX

Correspondence to: Dr. P. Pantazis, The Stehlin Foundation for Cancer Research, 1918 Chenevert Street, Houston, TX 77003, USA, Tel. No.: 713-756-5750; Fax No.: 713-756-5783.

Key Words: Leukemia cells, resistance, doxorubicin, camptothecin, cell differentiation.

0250-7005/95 $2.00+.40

Table I. *Percent of total cell population in various fractions of the cell cycle in untreated and verapamil-treated U-937/RD1000 cells.*

Histogram[a]	G0±G1	S	G2 + M	AP
A	40	50	2	8
B	30	57	3	10
C	13	41	33	14

[a]Percent of cells in each fraction was calculated by computer analysis of the histograms shown in Figure 8.

concentrations, respectively, that is, the increase in MRP synthesis precedes the increase in P-glycoprotein synthesis (25,26). Consequently, malignant cells and tumors resistant to DOX and having the MPR and MDR phenotype should be treated with drugs that can overcome these phenotypes. For example, camptothecin is an anticancer compound that overcomes expression of the MDR phenotype (27).

The plant alkaloid camptothecin (CPT) and the epipodophyllotoxin etoposide interfere with the mechanism of action of the nuclear enzymes topoisomerase I and II, respectively; by stabilizing the cleavable DNA-topoisomerase covalent complex, thus preventing subsequent DNA reunion and consequently producing DNA strand breaks which, if unrepaired, may eventually result in apoptosis (reviewed in refs. 28,29). CPT and its semi-synthetic derivative 9-nitro-CPT (9NC) have demonstrated high cytostatic and cytotoxic activity in a variety of human tumor cells *in vitro* and *in vivo* (reviewed in refs. 30,31). In general, the mechanism of action of CPT analogues and etoposide are very different from the mechanism of action of DOX. Therefore, in this report we have developed DOX-resistant human leukemia U-937 cell sublines *in vitro* and studied whether these cells have developed altered sensitivities to 9NC and etoposide and whether the responses correlate with proteins associated with resistance, regulation of apoptosis, and cell differentiation. The findings of these studies provide valuable information for development of protocols used in the chemotherapy of leukemias and other human malignancies.

Materials and Methods

Drugs. The anthracycline antibiotic DOX (Cetus; Emeryville, CA) and etoposide (VePesid; Bristol-Myers Squibb, Evansville, IN) were supplied as aqueous solutions. 9NC was prepared and purified from crude extract of CPT in our laboratory according to published procedures (32), and then used as a fine suspension in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI). Verapamil was from Sigma (St. Louis, MO). Treatment of cells with various compounds are described in the appropriate section of the text.

DOX-resistant cells. DOX-resistant (U-937/RD) cell sublines were developed from wild-type U-937 (U-937/WT) cells adapted to grow in media containing progressively increased concentrations of DOX. Thus, the U-937/RD40, U-937/RD200 and U-937/RD1000 cell sublines have been adapted to propagate in media containing 40 ng, 200 ng, and 1000 ng DOX per ml, respectively. The cells remained in drug-containing media for 4 additional months before they were used in the studies reported here or stored frozen in liquid nitrogen for future studies. Each cell subline was considered established when the following criteria were satisfied: (a) the number of viable cells in the culture was constantly above 95% as assessed by the trypan blue exclusion method, (b) no further variations were observed in the proliferation rates of continuously propagated cells, (c) no significant changes were detected in the relative DNA content of cell fractions as determined by flow cytometry, and (d) no further changes in morphological features were observed by microscopy of stained cells. U-937/WT and U-937/RD cells were maintained in RPMI 1640 medium (GIBCO; Grand Island, NY) supplemented with 10% bovine serum, glutamine, antibiotics, and the appropriate drug concentration. The cell cultures were incubated at 37°C in a humidified, 5% CO2-atmosphere.

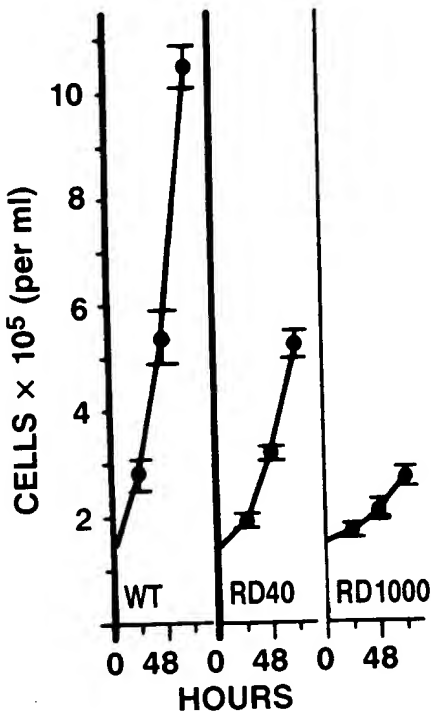


Figure 1. *Proliferation of U-937/WT and U-397/RD cells. Exponentially proliferating cells were pelleted by gentle centrifugation and resuspended at 1.5x105 cells/ml in 75% fresh media - 25% media conditioned by U-937/WT cells. Subsequently, the cultures received no other additive (U-937/WT) or doxorubicin at 40 ng/ml (U-937/RD40) and 1000 ng/ml (U-937/RD1000). Live cells were counted every 24 hr. WT, U-937/WT; RD40, U-937/RD40, and RD1000, U-937/RD1000.*

Microscopy and flow cytometry. Cytospin-prepared slides of cells were fixed, stained with Wright-Giemsa (Accustain; Sigma, St. Louis, MO), examined under a Zeiss microscope equipped with an electronically adjusted camera, and photomicrographed on Kodak 200 film. Relative DNA content of cells was determined by flow cytometry using an EPICS-ELITE Laser Flow Cytometer (Coulter Corp., Hialeah, FL) and analyzed with the aid of the MULTICYCLE program (Phoenix Flow Systems; San Diego, CA) that provides highly accurate evaluation of S-

Figure 2. *Morphology of stained cells. Exponentially proliferating U-937/WT (A), U-937/RD40 (B) and U-937/RD1000 (C) cells were pelleted on slides, stained and photomicrographed. Bar equals 20 nm (in C).*

phase of an asynchronous cell population (reviewed in ref. 33). The methodologies for microscopy and flow cytometry used in the present study have been used in several recent reports (34-39).

Western blot analysis. Cells were washed twice in PBS and then lysed in 200 µl of 1 mM EDTA, 0.2% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml E64, 10 µg/ml pepstatin, 1 µg/ml aprotinin, 20 µg/ml chymostatin, 50 µg/ml bestatin, 50 µg/l phosphoramidon, and 50 µg/ml antipan-dihydrochloride. The lysate was centrifuged at 12,500 xg for 10 min at 4°C, and the clarified supernatant was recovered. The protein concentration in the whole cell extract was determined using BioRad protein assay reagents. Fifty µg of protein were subjected to electrophoresis in a 12% polyacrylamide gel under non-reducing conditions. Afterwards, proteins were transferred onto nitrocellulose via electrophoresis in a Trans-Blot chamber (BioRad). Bcl-2 and Bax proteins were detected with a monoclonal mouse antibody that recognizes human Bcl-2 protein (Oncogene Science) and a polyclonal antibody that recognizes Bax protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The antibody-bound proteins were visualized by chemiluminescence (ECL-Western reagents: Amersham) according to the protocols provided by the manufacturers.

RNA analysis. Total RNA was prepared according to the procedure of Gough (40) and 50 µg were electrophoresed on a 1.2% agarose gel containing 6% formaldehyde. The RNA was transferred onto a nitrocellulose filter, immobilized by U.V. irradiation (Stratalinker; Stratagene, La Jolla, CA), and then incubated at 42°C for 4 hr in 5x Denhardt's solution containing 50% formamide, 0.5% SDS, and 100 µg/ml salmon sperm DNA. This was followed by an incubation at 42°C for 15 hr in 5x Denhardt's solution containing 50% formamide, 5x SSPE, 0.1% SDS, 100 µg/ml salmon sperm DNA, and 10^6 cpm/ml of radiolabelled probe. The filter was washed three times in 0.1x SSC buffer - 0.1% SDS at 60°C with a buffer change every 45 min. The washed filter was air-dried and exposed to X-ray films at -85°C. The DNA probe used was the 4.0-kb *Eco*R1 fragment of the human c-*fms* gene purified from the pc-*fms* 102 plasmid (41). The probe was radiolabelled with (α-^{32}P)dATP using random priming reagents purchased from Stratagene.

Tumorigenicity of cells. The ability of DOX-resistant cells to induce tumors in immunodeficient (nude) mice was tested as described (42). Briefly, about $2x10^7$ live cells were inoculated in each mouse, subcutaneously, and tumor growth was monitored daily by direct observation. Each cell type was tested in a group of six mice. Animals of the control group were inoculated with U-937/WT cells. In some instances, tumor specimens were surgically removed from anesthetized tumor-bearing mice and retransplanted in new mouse groups at six mice per group. Serial tumor transplantation in nude mice is a methodology that indicates tumorigenic ability, and tumor growth aggressiveness (43,44).

Results

Cell proliferation and DNA content. Figure 1 shows the proliferation curves of U-937/WT and U-937/RD cells. Proliferation of the U-937/RD cells was monitored while the cell cultures were propagated in media continuously containing DOX. It is apparent that the U-937/RD cells proliferate much slower than U-937/WT cells. The doubling times of the cells were estimated from the steepest slope of the curves to be 20±2 hr, 34±6 hr, and >100 hr for the U-937/WT, U-937/RD40, and U-937/RD1000 cells, respectively. The doubling times were estimated from four to six experiments for each cell subline, and while 95% or more of the cells were alive as assessed by the trypan blue exclusion method.

Studies of proliferation rates of the cell sublines were accompanied by analyses of relative DNA contents of the cells using flow cytometry. The mean DNA content in the cells in G0+G1, or DNA index (DI), for the U-937/WT and U-937/RD1000 cells are 1.45±0.06 and 1.76±0.03, respectively, following six determinations per cell subline. These results indicate an apparent increase in the DI of the U-937/RD1000 cells.

Morphology of doxorubicin-resistant cells. Figure 2 shows U-937/WT and DOX-resistant U-937 cells photo-micrographed at the same magnification. U-937/WT cells exhibit a fairly homogeneous size, small basophilic cytoplasm, and strongly stained reticular nuclei (A). However, U-937/RD40 (B) and U-937/RD1000 (C) cells are more enlarged than U-937/WT cells with increased ratio of cytoplasm to nucleus, and are finely granular. In addition, U-937/RD1000 cells are very basophilic (C).

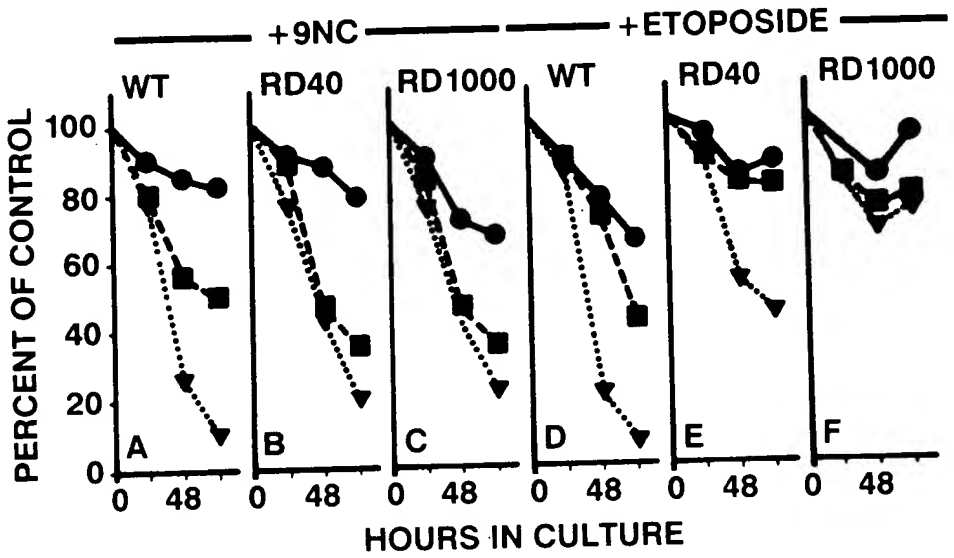


Figure 3. *Effect of topoisomerase-directed drugs on cell proliferation. Cultures containing equal number of cells received 9NC at 0.005 μM (●), 0.02 μM (■) and 0.1 μM (▼) or etoposide at 0.05 μM (●), 0.2 μM (■) and 1 μM (▼). Control cell culture received no drug treatment. Cells were counted every 24 hr and plotted as percent of the cell number in the control culture that was taken to be 100%.*

Treatment of resistant cells with topoisomerase-directed drugs. In recent reports, we have shown that development of U-937 cell sublines resistant to 9NC is accompanied by increased sensitivity to etoposide (45), and reversibly, increased resistance to etoposide renders the cells more sensitive to 9NC (unpublished data). Therefore, in this section, we have studied whether U-937/RD40 and U-937/RD1000 cells exhibit altered sensitivities to the topo I-directed 9NC and topo II-directed etoposide. Comparison was made with the sensitivities of U-937/WT cells to these drugs. All three cell types were treated with the same 9NC or etoposide concentration and inhibition of cell proliferation was estimated as percent of the proliferation rate of untreated cells that was considered to be 100%. The results of these studies are graphically shown in Figure 3. U-937/WT and U-937/RD cells are about equally sensitive to 9NC, but U-937/RD cells are significantly more resistant than U-937/WT cells to etoposide. In fact, treatment with 1000 nM of etoposide for 72 hr resulted in death of virtually all U-937/WT cells, while 60% or more of the U-937/RD cells were still alive at 72 hr of drug treatment. However, continuous presence of etoposide in cultures of U-937/RD cells resulted only in a partial inhibition of cell proliferation, since after 96 hr, etoposide-treated and untreated U-937/RD cells exhibited similar proliferation rates (results not shown).

In parallel studies, we monitored perturbations in the cell cycle of cells treated with 9NC or etoposide for various periods of time. The cell cycle changes were monitored by detecting changes in the relative DNA content of cells using flow cytometry. The results (histograms) are shown in Figure 4. No apparent changes were observed in the cell cycle of U-937/WT cells treated with 20 nM 9NC for 24 hr (group A, histogram D), but there was an increased accumulation of cells at G2 after 72 hr and 120 hr of treatment (histograms E and F, respectively). A fraction of apoptotic cells (AP) appeared only after a 120 hr-period of treatment with 20 nM 9NC (histogram F). However, 20 nM 9NC induced dramatic perturbations in the cell cycle of U-937/RD40 (group B, histograms D,E,F) and U-937/RD1000 cells (group C, histograms D,E,F) with appearance of a large apoptotic fraction after 72 hr of treatment (histogram E). The AP fraction increased further after 120 hr of treatment with 20 nM 9NC (histograms F). A 9NC-concentration of 100 nM induced accumulation of virtually all U-937/WT cells at late-S/G2 (group A, histogram H), followed by apoptosis during further treatment (group A, histogram I). However, the same 9NC concentration induced accumulation of the U-937/RD1000 cells in the S-phase rather than in late-S/G2, with an apoptotic fraction (group C, histograms H and I) smaller than that observed in U-937/RD40 cells (group B, histograms H and I).

In this section, we also monitored perturbations in the cell cycle of U-937/WT and U-937/RD cells treated with the topo II-directed drug etoposide. The results are included in Figure

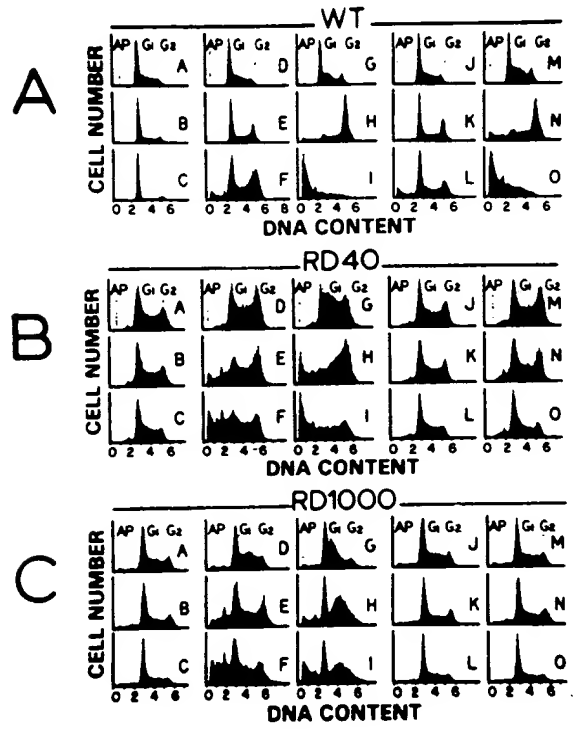


Figure 4. *Perturbations in the cell cycle of cells treated with topoisomerase-directed drugs. Group A: U-937/WT cells; Group B: U-937/RD40 cells; Group C: U-937/RD1000 cells: histograms A,B,C,: untreated cells; histograms D,E,F: cells +20 nM 9NC: histograms G,H,I: cells +100 nM 9NC; histograms J,K,L: cells +200 nM etoposide; histograms M,N,O: cells +1000 nM etoposide. Control and treated cells at 24 hr (A,D,G,J,M), 72 hr (B,E,H,K,N) and 120 hrs (C,F,I,L,O). G1 = G0 + G1 ; G2= G2 + M; AP = apoptotic fraction.*

Figure 5. *Detection of Bax. Total cell protein was prepared from U-937/WT, U-937/RD40 and U-937/RD1000 cells and subjected to Western blot analysis using an antibody specific for Bax protein.*

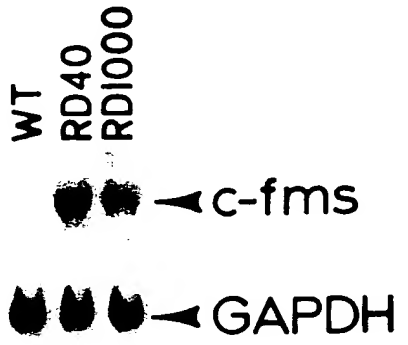


Figure 6. *Detection of c-fms mRNA. Total cellular RNA was prepared from U-937/WT, U-937/RD40 and U-937/RD1000 cells and subjected to Northern blot analysis for presence of c-fms and GAPDH mRNAs.*

4. It can be seen that 200 nM etoposide induced arrest of some cells in G2, and subsequent apoptosis during treatment for 72 hr and 120 hr (group A, histograms K and L). On the other hand, the same etoposide concentration had no significant effect on the cell cycle of U-937/RD40 (group B, histograms J,K,L) and U-937/RD1000 (group C, histograms J,K,L) cells. The only change observed is a continuous decrease in the S- and G2-fractions, apparently as a result of the decrease in proliferation rates of the cells. Further, a much higher etoposide concentration of 1000 nM induced a dramatic accumulation of cells at G2, appearance of apoptotic cells, and a simultaneous decrease in the G1-fraction (group A, histograms M and N), while longer exposure to this drug concentration resulted in apoptosis in almost all wild-type cells (group A, histogram O). In contrast, 1000 nM of etoposide resulted in a small increase in the apoptotic fraction of U-937/RD40, but not U-937/RD1000 cells. These results indicate that U-937/RD1000 cells are more resistant than U-937/RD40 cells to etoposide treatments. Taken together the findings in this section, the conclusion is that U-937/RD cells are sensitive to 9NC, like U-937/WT cells, but less sensitive than U-937/WT cells to etoposide.

Synthesis of proteins regulating apoptosis. The proteins Bcl-2 and Bax have been associated with one of the mechanisms that regulates apoptosis in cells exposed to various cytotoxic agents (16,17,46,47). Therefore, steady-state synthesis of Bcl-2 and Bax was investigated in wild-type and DOX-resistant U-937 cells by Western analysis. Very little Bcl-2 was detected in U-937/WT and synthesis of this protein was not increased in U-937/RD cells, whereas a high level of Bax protein was present in the DOX-resistant cells, but only a low level, presumably basal level, synthesis was observed in U-937/WT cells (Figure 5).

Detection of c-fms mRNA in DOX-resistant cells. Molecular

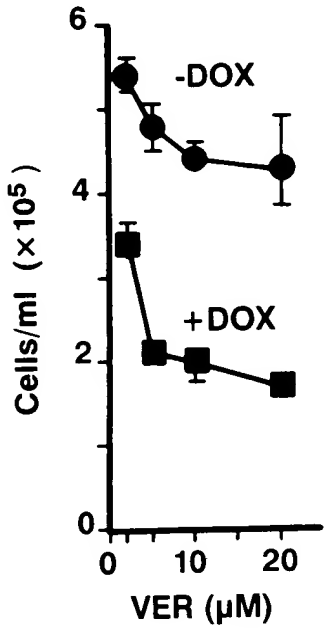


Figure 7. *Proliferation of U-937/RD1000 cells in presence of verapamil. U-937/RD1000 cells were grown in continuous presence of 1000 ng/ml DOX. Exponentially growing cells were removed and equal number of cells were transferred to fresh culture media containing verapamil alone (●) or verapamil and DOX (■). Live cells were counted at 72 hr of drug treatment.*

hybridization studies revealed no detectable presence of c-*fms* mRNA in U-937/WT cells, whereas this mRNA species was readily detected in DOX-resistant cells (Figure 6). There was no significant difference in the amounts of c-*fms* mRNA detected in U-937/RD40 and U-937/RD1000 cells despite the fact that U-937/RD1000 cells are 25 times more resistant than U-937/RD40 cells to DOX. Detection of similar amounts of GAPDH mRNA in U-937/WT and U-937/RD cells confirmed that equal amounts of total cellular RNA were subjected to analysis for presence of c-*fms* mRNA.

Acquired resistance to DOX is partially associated with P-glycoprotein. It has been shown that synthesis of P-glycoprotein confers resistance to cells against several anticancer drugs derived from plants, and that the action of P-glycoprotein can be overcome in drug-resistant cells in the presence of the compound verapamil (reviewed in refs. 48,49). Therefore, in this section, we have used verapamil to investigate whether resistance to DOX in U-937/RD cells is associated with P-glycoprotein. Verapamil was used at various concentrations, the maximal being 20 μM. In preliminary studies, it was determined that verapamil concentrations of 20 μM or less do not affect proliferation of U-937/WT cells. For each verapamil concentration used, three aliquots containing equal number of cells were removed from a culture of exponentially grown cells and gently centrifuged. One cell pellet was resuspended in fresh media containing the appropriate DOX concentration (culture A); the second cell pellet was suspended and washed in PBS, then resuspended in fresh media with no DOX but containing verapamil (culture B); the third cell pellet was resuspended in media containing DOX and verapamil (culture C). All cultures were incubated at 37°C for 72 hr, then aliquots were removed and live cells were scored as an indicator of changes in the proliferation rates. The results of the studies with U-937/RD1000 cells are shown in Figure 7. Presence of verapamil inhibited cell proliferation both in absence and presence of DOX, but the verapamil-induced inhibition was more dramatic in presence of DOX, apparently because DOX was more active in presence of verapamil.

Studies of cell proliferation were accompanied by observations on perturbations in the cell cycle reflected by changes in the relative DNA content and determined by flow cytometry. Histograms of verapamil-untreated and treated U-937/RD1000 cells are shown in Figure 8. Compared to cells exposed to DOX alone (histogram A), there was an apparent increase in the S-fraction of cells treated with verapamil alone (histogram B), whereas exposure to both drugs, DOX and verapamil, resulted in accumulation of a large number of cells in late S- and M-phases, and generation of a readily detected apoptotic fraction (histogram C). Similar results were obtained with both U-937/RD40 and U-937/RD1000 cells, and therefore, only results with U-937/RD1000 cells are shown in Figures. 7 and 8. Taken together, these findings suggest that verapamil results in a more extensive decrease in cell proliferation in presence rather than in absence of DOX, and that part of this decrease is due to death of a portion of the cultured cells.

Tumorigenic ability of cells. We have recently shown that U-937/WT cells xenografted in nude mice induce visible tumors 7 to 10 days postinoculation (33). Therefore, in this section, we have tested the tumorigenic ability of U-937/RD cells xenografted in nude mice. Following daily examinations, visible tumors appeared 8-10 days in animals inoculated with U-937/WT cells in agreement with previous observations (33). Of the six animals inoculated with U-937/RD40 cells, four developed small tumors. Samples removed from these tumors were transplanted into new groups of mice or subjected to isoenzyme analysis to assess the human origin of tumors. None of the transplanted samples induced tumors two months after transplantation, whereas transplanted U-937/WT (control) biopsies induced tumors 6-8 days after transplantation. Similarly, U-937/RD1000 cells induced tumors in two of six inoculated mice, with no induction of tumors following serial transplantation. The human origin of the transplanted tumors was assessed by isoenzyme analysis. Therefore, the results of these studies indicate that U-937/RD cells lack tumorigenic ability.

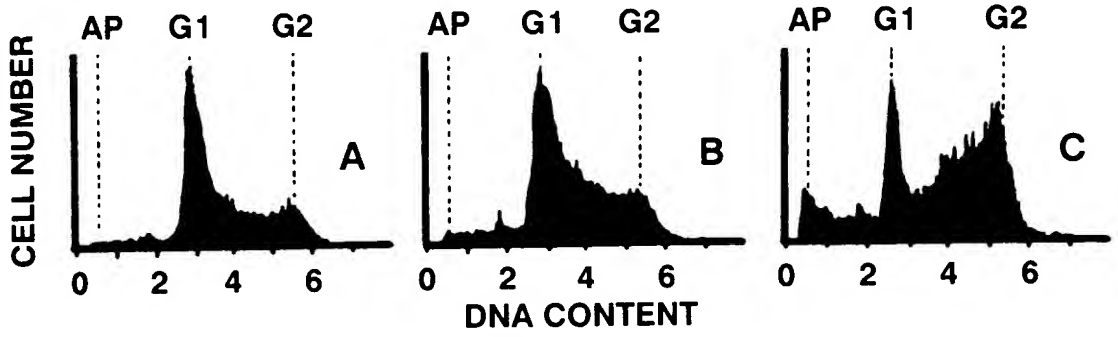


Figure 8. *Perturbations in the cell cycle of U-937/RD1000 cells treated with verapamil. U-937/RD1000 cells were treated as in Figure 7 for 72 hr and analyzed for relative DNA content by flow cytometry. The histograms shown are from cells treated with DOX alone (A), 20 mM verapamil alone (B), and DOX + verapamil (C).*

Discussion

Acquisition of resistance to DOX by cancer cells or tumors dictates that these cells or tumors must be treated with another agent that acts via a mechanism different than that of DOX. The choice of a chemotherapeutic agent that will effectively target DOX-resistant cells will be more successful if we could identify events and/or properties associated with development of DOX-resistance.

In this study, we have presented evidence that increases in resistance to DOX correlate with slower proliferation rates of the resistant cells. These changes in the length of the cell doubling time could be associated with differentiation of these cells. Indeed, loss of the round appearance of the nuclei is an indicator that the DOX-resistant U-937 cells are more differentiated than the U-937/WT cells. In addition, c-*fms* mRNA was detected in U-937/RD but not U-937/WT cells. c-*fms* mRNA codes for the monocyte colony-stimulating factor-1 receptor, CSF-1R (50), which is primarily present in peripheral blood monocytes, tissue macrophages and their committed bone marrow progenitors (51), and leukemia cells induced to differentiate along the monocytic lineage *in vitro* (52,53). Finally, induction of differentiation, *i.e.*, maturation, correlates well with the decreased ability or inability of U-937/RD cells to induce tumors when xenografted in nude mice. In contrast, U-937/WT cells are highly tumorigenic when xenografted in nude mice, with visible tumors appearing 7 to 10 days after cell inoculation (54). In this regard, it is well documented that in the majority of the tumors studied, the more differentiated the human cells, the less they take in nude mice (55). In conclusion, the findings described in this report show that development of resistance to DOX by U-937 cells is intimately associated with induction of differentiation of these cells along the monocytic lineage.

DOX indirectly interferes with the mechanisms of action of topoisomerase I and II (9,13) and this leads to altered levels of DNA-topoisomerase cleavable complexes that are targeted by topoisomerase-directed drugs. Therefore, we have investigated whether DOX-resistant U-937 cells exhibit different sensitivities than U-937/WT cells to the topoisomerase I- and II-directed drugs 9NC and etoposide, respectively. Our studies showed that U-937/WT and U-937/RD cells exhibited similar sensitivity to 9NC, that is, the sensitivity to 9NC remains practically unaltered in U-937 cells that developed resistance to DOX. In contrast, the U-937/RD cells demonstrate much less sensitivity than U-937/WT cells to etoposide, in agreement with a previous report that development of resistance to DOX in mouse leukemia L1210 and P388 cells correlates with a decrease in topo II activity (56,57). Pertinent to these findings are reports that, unlike camptothecin, DOX and etoposide cannot overcome overexpression of P-glycoprotein that confers resistance in malignant cells to several unrelated anticancer drugs (19,58-60). Further, low concentrations of verapamil had little or no effect on the proliferation rate of U-937/RD cells in absence of DOX, but the same drug concentrations resulted in increased inhibition or cessation of cell proliferation in presence of DOX, and this was followed by induction of apoptosis in a portion of cells in the culture. Apparently, verapamil circumvented P-glycoprotein-induced neutralization of the cytotoxic effect of DOX. Because of these results we have concluded that part of the acquired insensitivity to DOX correlates with presence of P-glycoprotein. We have not investigated whether U-937/RD cells synthesize the multidrug resistance-associated protein, MRP. However, it has been recently reported that induction of MRP overexpression occurs before that of the P-glycoprotein gene (25).

Also of interest is our finding that DOX-resistant cells overproduce Bax, whereas this protein is not readily detected in U-937/WT cells. Molecular and cellular studies have

shown that the anti-apoptotic proteins Bcl-2 and Bcl-x must heterodimerize with Bax to regulate apoptosis (17,61), and overexpression of Bax sensitizes the cells to induction of apoptosis (47). It has been proposed that the ratio of Bcl-2 or Bcl-x to Bax determines the cell's susceptibility to a stimulus of apoptosis (17,62). Further, it has been recently reported that constitutive overexpression of Bcl-X_L, but not Bcl-2, correlates with development of resistance in U-937 cells to DOX, vincristine, and radiation (15). The findings in this report show marked differences in the levels of Bax synthesis in U-937/WT and U-937/RD cells. However, these cells differ in susceptibility to etoposide but not to 9NC, and, therefore, it is possible that Bax overproduction correlates with resistance of U-937/RD cells only to certain chemotherapeutic agents. Further, we have shown that U-937/RD cells are more differentiated than U-937/WT cells along the monocytic lineage, and therefore a possibility is that Bax overexpression correlates with the stage of cell maturation. In contrast, a marked decrease in Bcl-2 mRNA and protein levels was observed in monocytic as well as granulocytic differentiated HL-60 cells (63). There is no experimental evidence to correlate overproduction of Bax with cell differentiation and/or resistance to various chemotherapeutic agents. Such studies are underway in our laboratory.

In conclusion, the findings reported here show that development of U-937 cell resistance to DOX is a process that involves more than one factor. However, the clinical importance is that 9NC is the drug of choice for the effective treatment of U-937 cells, and possibly other cell types and tumors, that develop resistance to DOX.

Acknowledgements

We thank B. Harris for typing the manuscript. This work was supported by funds from The Stehlin Foundation and The Friends of the Stehlin Foundation.

References

1 *In:* The Anticancer Drugs (Pratt WB, Ruddon RW, Ensminger WD, Maybaum J, eds.), chapter 7: Noncovalent DNA-binding drugs, pp. 155-182. Oxford University Press, New York, 1994.

2 Riggs CE. Antitumor antibiotics and related compounds. *In:* The Chemotherapy Source Book (Perry MC, ed.), pp. 318-358. Williams & Wilkins, Baltimore, MD 1992.

3 Powis G: Metabolism and reactions of quinoid anticancer agents. Pharmacol Ther *35:* 57-162, 1987.

4 Bachur NR: Adriamycin-daunorubicin cellular pharmacodynamics. Biochem Pharmacol *23:* 207-216, 1974.

5 Vig BK: Mutagenic effects of some anticancer antibiotics. Cancer Chemother Pharmacol *3:* 143-160, 1979.

6 Mimnaugh EG, Trush MA, Bhatnagar M, Gram TE: Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin. Biochem Pharmacol *34:* 847-856, 1985.

7 Kanter PM, Schwartz HS: Adriamycin-induced DNA damage in human leukemia cells. Leukemia Res *3:* 277-283, 1979.

8 Myers CE, McGuire WP, Liss RH, Ifrim K, Grotzinger K, Young, RC: Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. Science *197:* 165-167, 1977.

9 Tewey KM, Chen GL, Nelson EM, Liu LF: Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalial DNA topoisomerase II. J Biol Chem *259:* 9182-9187, 1984.

10 Vosberg H-P: DNA topoisomerases: Enzymes that control DNA conformation. Current Topics Microbiol Immunobiol *114:* 19-102, 1986.

11 Sugimoto Y, Tsukahara S, Oh-Hara T, Isoe T, Tsunio T: Decreased expression of topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. Cancer Res *50:* 6925-6930, 1990.

12 Wasserman K, Markovits J, Jaxel C, Capranico G, Kohn K, Pommier Y: Effects of morpholinyl doxorubicins, doxorubicin and actinomycin D on mammalian DNA topoisomerases I and II. Mol Pharmacol *38:* 38-48, 1990.

13 Foglesong PD, Reckford C, Swink S: Doxorubicin inhibits human DNA topoisomerase I. Cancer Chemother Pharmacol *30:* 123-125, 1992.

14 Fornari FA, Jarvis WD, Grant S, Orr MS, Randolph JK, White FKH, Mumaw VR, Lovings ET, Freeman RH, Gewirtz DA: Induction of differentiation and growth arrest associated with nascent (monoligosomal) DNA fragmentation and reduced by c-*myc* expression in MCF-7 human breast tumor cells after continuous exposure to a sublethal concentration of doxorubicin. Cell Growth Diff *5:* 723-733, 1994.

15 Datta R, Manome Y, Taneja N, Boise LH, Weichselbaum R, Thompson CB, Slapak CA, Kufe D: Overexpression of Bcl-X_L by cytotoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. Cell Growth Diff *6:* 363-370, 1995.

16 Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nuñez G, Thomson CB: Bcl-X, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74:* 597-608, 1993.

17 Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74:* 609-619, 1993.

18 Sato T, Hanada M, Bodrug S, Irie S, Iwana N, Boise LN, Thomson CB, Golemis E, Fong L, Wang HG, Reed CR: Interactions among members of the bcl-2 protein family analyzed with a yeast two-hybrid system. Proc Natl Acad Sci USA *91:* 9238-9242, 1994.

19 Endicott JA, Ling V: The biochemistry of P-glycoprotein-mediated multidrug resistance. Ann Rev Biochem *58:* 137-171,1989.

20 Beck WT: Mechanisms of multidrug resistance in human tumor cells. The roles of P-glycoprotein, DNA topoisomerase II, and other factors. Cancer Treat Rep *17:* 11-20, 1990.

21 Gottesman MM, Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem *62:* 385-427, 1993.

22 Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG: Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science *258:* 1650-1654, 1992.

23 Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG: Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res *54:* 357-361, 1994.

24 Shen J, Hughes C, Chao C, Cai J, Bartels C, Gessner T, Subjeck J: Coinduction of glucose regulated proteins and doxorubicin resistance in Chinese hamster cells. Proc Natl Acad Sci USA *84:* 3278-3282, 1987.

25 Slapak CA, Mizunuma N, Kufe DW: Expression of the multidrug resistance associated protein and P-glycoprotein in doxorubicin-selected human myeloid leukemia cells. Blood *84:* 3113-3121, 1994.

26 Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deely RG, Cole SPC, Sehested M: Sequential coexpression of the multidrug resistance genes MRP and *mdr*1 and their products in VP-16 (etoposide)-

selected H69 small cell lung cancer cells. Cancer Res *55:* 459-462, 1995.

27 Chen AY, Potmesil M, Wall ME, Wani MC, Liu LF: Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. Cancer Res *51:* 6039-6044, 1991.

28 Pommier Y, Tanizawa A, Kohn KW: Mechanisms of topoisomerase I inhibition by anticancer drugs. Adv Pharmacol *29B:* 73-92, 1994.

29 Osheroff N, Corbett AH, Robinson MJ: Mechanisms of action of topoisomerase II-targeted antineoplastic drugs. Adv Pharmacol *29B:* 105-126, 1994.

30 Pantazis P: The water-insoluble camptothecins: Promising anticancer drugs. Cancer J *8:* 119-123, 1995.

31 Pantazis P: The water-insoluble camptothecin analogues: Promising drugs for the effective treatment of haematological malignancies. Leukemia Res 1995 (in press).

32 Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA: Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata. J Am Chem Soc *88:* 3888-3890, 1966.

33 Jayat C, Ratinaud MH: Cell cycle analysis by flow cytometry: Principles and applications. Biol Cell *78:* 15-25, 1993.

34 Pantazis P, Mendoza JT, DeJesus A, Rubin E, Kufe D, Giovanella BC: Partial characterization of human leukemia U-937 cell sublines resistant to 9-nitro-camptothecin. Eur J Haematol *53:* 135-144, 1994.

35 Pantazis P, Mendoza J, DeJesus A, Early J, Shaw M, Giovanella B: Development of resistance to 9-nitro-camptothecin by human leukemia U-937 cells in vitro correlates with altered sensitivities to several anticancer drugs. Anti-Cancer Drugs *5:* 473-479, 1994.

36 Pantazis P, Hinz HR, Mendoza JT, Kozielski AJ, Williams LJ, Stehlin JS, Giovanella BC: Complete inhibition of growth followed by death of human malignant melanoma cells in vitro and regression of human melanoma xenografts in immunodeficient mice by camptothecins. Cancer Res *52:* 3980-3987, 1992.

37 Pantazis P, Early JA, Kozielski AJ, Mendoza JT, Hinz HR, Giovanella BC: Regression of human breast carcinoma tumors in immunodeficient mice treated with 9-nitro-camptothecin: Differential response of nontumorigenic and tumorigenic human breast cells in vitro. Cancer Res *53:* 1577-1582, 1993.

38 Pantazis P, Kozielski AJ, Mendoza JT, Early JA, Hinz HR, Giovanella BC: Camptothecin derivatives induce regression of human ovarian carcinomas grown in nude mice and distinguish between non-tumorigenic and tumorigenic cells in vitro. Int J Cancer *53:* 863-871, 1993.

39 Pantazis P, Early JA, Mendoza JT, DeJesus AR, Giovanella BC: Cytotoxic efficacy of 9-nitrocamptothecin in the treatment of human malignant melanoma cells *in vitro.* Cancer Res *54:* 771-776, 1994.

40 Gough NM: Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Analyt Biochem *173:* 93-95, 1988.

41 Coussens L, Van Beveren C, Smith D, Chen E, Mitchell RL, Isacke CM, Verma IM, Ullrich A: Structural alteration of viral homologue of receptor proto-oncogene fms at carboxyl terminus. Nature *320:* 277-280, 1986.

42 Verschraegen CF, Giovanella BC, Mendoza JT, Kozielski AJ, Stehlin JS: Specific organ metastases of human melanoma cells injected into the arterial circulation of nude mice. Anticancer Res *11:* 529-536, 1991.

43 Giovanella BC, Fogh J: Present and future trends in invesitgations with the nude mouse as a recipient of human tumor transplants. *In:* The Nude Mouse in Experimental and Clinical Research, (Fogh J and Giovanella BC, eds.), Academic Press, New York, NY, 1978, pp. 281-312.

44 Fodstad O: Transplantability of human tumors. *In:* The nude mouse in oncology research, (Boven E, Winograd B, eds.), CRC Press, Inc., Boca Raton, FL, 1991, pp. 65-80.

45 Pantazis P, Vardeman D, Mendoza J, Early J, Kozielski A, DeJesus A, Giovanella BC: Sensitivity of camptothecin-resistant human leukemia cells and tumors to anticancer drugs with diverse mechanisms of action. Leukemia Res *19:* 43-55, 1995.

46 Sentman CL, Shutter JR, Hockenberry D, Kanagawa O, Korsmeyer SJ: Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell *67:* 879-888, 1991.

47 Reed JC: Bcl-2 and regulation of programmed cell death. J Cell Biol *124:* 1-6, 1994.

48 Chabner BA, Wilson W: Reversal of multidrug resistance. J Clin Oncol *9:* 4-6, 1991.

49 Ford JM, Hait WN: Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev *42:* 155-199, 1991.

50 Sherr CJ, Rettenmeier CW, Sacco R, Roussel MF, Look AT, Stanley ER: The c-fms protooncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell *41:* 665-676, 1985.

51 Nienhuis AW, Bunn HF, Turner PH, Venkat Gopal T, Nash WG, O'Brien SJ, Sherr CJ: Expression of the human c-fms protooncogene in hematopoietic cells and its deletion in the 5q-syndrome. Cell *42:* 421-428, 1985.

52 Sariban E, Mitchell T, Kufe D: Expression of c-fms protooncogene during human monocytic differentiation. Nature *316:* 64-66, 1985.

53 Pantazis P, Kharbanda S, Goustin AS, Galanopoulos T, Kufe D: Coexpression of the genes for platelet-derived growth factor B-chain receptor and macrophage colony-stimulating factor I receptor during monocytic differentiation. Proc Natl Acad Sci USA *88:* 2481-2485, 1991.

54 Pantazis P, Mendoza JT, Kozielski AJ, Natelson EA, Giovanella BC: 9-nitro-camptothecin delays growth of U-937 leukemia tumors in nude mice and is cytotoxic or cytostatic for human myelomonocytic leukemia lines in vitro. Eur J Haematol *50:* 81-89, 1993.

55 Friedman VH, Shin S: Use of nude mice for studies on the tumorigenicity of animal cells. *In:* The Nude Mouse in Experimental and Clinical Research, (Fogh J, Giovanella BC, ed.), Academic Press, New York, NY, 1978, pp. 353-384.

56 Ganapathi R, Grabowski D, Ford J, Heiss C, Kerrigan D, Pommier Y: Progressive resistance to doxorubicin in mouse leukemia L1210 cells with multidrug resistance phenotype: Reduction in drug-induced topoisomerase II-mediated DNA cleavage. Cancer Commun *1:* 217-224, 1989.

57 Deffie AM, Batra JK, Goldberger GJ: Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines. Cancer Res *49:* 58-62, 1989.

58 Chen AY, Potmesil M, Wall ME, Wani MC and Liu LF: Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. Cancer Res *51:* 6039-6044, 1991.

59 *In:* Anticancer Drugs (Pratt WB, Ruddon RW, Ensminger WD, Maybaum J, eds), chapter 4: Resistance to anticancer drugs, New York, Oxford University Press, 1994, pp. 50-66.

60 Nooter K, Sonneveld P: Clinical relevance of P-glycoprotein expression in haematological malignancies. Leukemia Res *18:* 233-243, 1994.

61 Yin X-M, Oltvai ZN, Korsmeyer SJ: BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature *369:* 321-323, 1994.

62 Oltvai ZN, Korsmeyer SJ: Checkpoints of deuling dimers foil death wishes. Cell *79:* 189-192, 1994.

63 Delia D, Aiello A, Soligo D, Fontanella E, Melani C, Pezzella F, Pierotti MA, Della Porta G: bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. Blood *79:* 1291-1298, 1992.

Received July 4, 1995
Accepted July 24, 1995

STIC-ILL

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic stem cells in culture.
SO SCIENCE, (1994 Aug 19) 265 (5175) 1098-101.
Journal code: UJ7; 0404511. ISSN: 0036-8075.

1

local mast cell replacement (7). Triggering of mast cells could result in the release of preformed mediators, thereby increasing vascular permeability, activating complement, and stimulating the local adhesion and migration of neutrophils. Resident macrophages, dendritic cells, or neutrophils may serve a similar role in triggering a chemotactic cascade. FcR cross-linking by immune complexes on either of these cells may directly or in synergy with complement receptors result in the activation of complement components, along with known pro-inflammatory mediators, and thereby set off the cascade of events that culminates in the dramatic sequelae of the inflammatory response. In either case, inhibition of FcR cross-linking by immune complexes can be expected to attenuate the inflammatory response by targeting the initiation of the cascade rather than its propagation and amplification. Targeting of these receptors in autoimmune diseases represents another potentially potent therapeutic approach to preventing tissue injury by immune complexes.

REFERENCES AND NOTES

1. M. Arthus, *C. R. Soc. Biol.* **55**, 817 (1903).
2. C. G. Cochrane and A. Janoff, in *The Inflammatory Process*, B. W. Zweifach, L. Grant, R. T. McCluskey, Eds. (Academic Press, 1974), vol. III, chap. 3; Z. Ovary, *Int. Arch. Allergy Appl. Immunol.* **69**, 385 (1982).
3. D. T. Fearon and W. W. Wong, *Annu. Rev. Immunol.* **1**, 243 (1983); D. H. Perlmutter and H. R. Colten, *ibid.* **4**, 231 (1986).
4. J. T. Culbertson, *J. Immunol.* **29**, 29 (1935); P. R. Cannon and L. E. Marshall, *ibid.* **40**, 127 (1941); E. E. Fischel and E. A. Kabat, *ibid.* **55**, 337 (1947); B. Benacerraf and E. A. Kabat, *ibid.* **64**, 1 (1950); C. G. Cochrane and W. O. Weigel, *J. Exp. Med.* **108**, 591 (1958); C. A. Stetson, *ibid.* **94**, 349 (1951); J. H. Humphrey, *Br. J. Exp. Pathol.* **36**, 268 and 283 (1955); C. G. Cochrane, W. O. Weigle, F. J. Dixon, *J. Exp. Med.* **110**, 481 (1959); P. A. Ward and C. G. Cochrane, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 509 (1964).
5. J. V. Ravetch and J. P. Kinet, *Annu. Rev. Immunol.* **9**, 457 (1991); J. V. Ravetch, *Cell*, in press.
6. T. Takai, M. Li, D. Sylvestre, R. Clynes, J. V. Ravetch, *ibid.* **76**, 519 (1994).
7. Y. Zhang, B. F. Ramos, B. A. Jakschik, *J. Clin. Invest.* **88**, 841 (1991); *Science* **258**, 1957 (1992).
8. D. L. Sylvestre and J. V. Ravetch, unpublished results.
9. Evans blue (20 mg/ml) was added to the intravenous injectate: human serum albumin was iodinated by the lactoperoxidase method, and 10^6 cpm were included in each intravenous injection.
10. P. P. Bradley, D. A. Priebat, R. D. Christensen, G. Rothstein, *J. Invest. Dermatol.* **78**, 206 (1982).
11. R. M. W. De Waal, G. Schrijver, M. J. J. T. Bogman, K. J. M. Assmann, R. A. P. Koene, *J. Immunol. Methods* **108**, 213 (1988).
12. C3bi-coated SRBCs were derived by incubating SRBCs in phosphate-buffered saline (PBS) with IgM to SRBC for 30 min at 4°C; they were then incubated in veronal-buffered saline for 30 min at 37°C with serum derived from DBA mice, a C5-deficient strain.
13. A. C. Issekutz and T. B. Issekutz, in *Methods Enzymol.* **162**, 301 (1988).
14. C. G. Cochrane, J. J. Muller-Eberhard, B. S. Aikin, *J. Immunol.* **105**, 55 (1970).
15. Mice were injected intraperitoneally with 1 ml of 5% thioglycollate. Four hours later, the peritoneum was lavaged with PBS containing 0.1% fetal calf serum. The cellular material was then stained with acid hematoxylin.
16. Anti-OVA was iodinated by the lactoperoxidase method; 1.5×10^6 cpm was included in each intradermal injection.
17. D. Dombrowicz, V. Flamand, K. K. Brigman, B. H. Koller, J. P. Kinet, *Cell* **75**, 969 (1993).
18. D. L. Sylvestre and J. V. Ravetch, unpublished results.
19. E. S. Schulman, *Crit. Rev. Immunol.* **13**(1), 35 (1993); S. J. Galli, *N. Engl. J. Med.* **328**, 257 (1993); J. S. Pober, M. R. Slowik, L. G. DeLuca, A. J. Ritchie, *J. Immunol.* **150**, 5114 (1993); B. Benacerraf, R. T. McCluskey, D. Patras, *ibid.* **35**(1), 75 (1959).

20. We thank A. Aderem, P. Model, C. Homcy, B. Perussia, and T. Manser and others for discussion; J.-P. Kinet for providing FcεRIγ-deficient mice; D. Myers for assistance with histopathological interpretation; C. Rocco for slide preparation; N. Leibel for technical assistance; C. Chiang for figure designs; and C. Ritter for manuscript preparation. Supported by grant numbers AI34662 (J.V.R.) and AI01067 (D.S.) from the National Institute of Allergy and Infectious Diseases. All protocols used in the care and experimentation of mice were in accordance with NIH and institutional guidelines. We dedicate this study to the memory of Zanvil Cohn, an eminent scientist, teacher, and good friend whose contributions to our understanding of the inflammatory response were profound and whose insights we will sorely miss.

18 April 1994; accepted 20 June 1994

Generation of Lymphohematopoietic Cells from Embryonic Stem Cells in Culture

Toru Nakano, Hiroaki Kodama, Tasuku Honjo

An efficient system was developed that induced the differentiation of embryonic stem (ES) cells into blood cells of erythroid, myeloid, and B cell lineages by coculture with the stromal cell line OP9. This cell line does not express functional macrophage colony–stimulating factor (M-CSF). The presence of M-CSF had inhibitory effects on the differentiation of ES cells to blood cells other than macrophages. Embryoid body formation or addition of exogenous growth factors was not required, and differentiation was highly reproducible even after the selection of ES cells with the antibiotic G418. Combined with the ability to genetically manipulate ES cells, this system will facilitate the study of molecular mechanisms involved in development and differentiation of hematopoietic cells.

The mechanisms of determination and differentiation that lead to the formation of hematopoietic cells from the inner cell mass of blastocysts through mesodermal cells are still unelucidated in spite of identification of numerous hematopoietic growth factors. Hematopoietic differentiation of ES cells can be induced in vitro (*1–3*), but these systems require formation of complex embryoid structures or addition of exogenous growth factors, or both (*1–3*). Other limitations are the inability to dissect the developmental processes from ES cells to blood cells and the lack of simultaneous induction of both myeloid and lymphoid lineage cells. To overcome these limitations, we tested whether coculture of ES cells on stromal cells without exogenous growth factors might induce lymphohematopoietic differentiation. We initially tried the stromal cell lines ST2, PA6, and RP0.10 (*3*, *4*), but these cell lines gave rise almost exclusively to macrophages.

Because M-CSF might be responsible for the preferential differentiation of ES cells into the monocyte-macrophage lineage (*5*), we examined the differentiation-inducing activity of the OP9 stromal cell line. This line was established from newborn calvaria of the $(C57BL/6\times C3H)F_2$-*op*/*op* mouse that lacks functional M-CSF because of a mutation in the M-CSF gene (*6*, *7*). D3 ES cells established from the 129/Sv mouse strain (*1*) produced two types of colonies 5 days after transfer onto OP9 cells; one was typical of an undifferentiated ES cell colony (Fig. 1A) and the other had features of differentiated mesoderm-like colonies which consisted of $\sim 10^3$ adherent blastic cells larger than D3 cells (Fig. 1B) (*8*). The whole culture was then trypsinized and passed onto fresh OP9 cells (*9*). Clusters consisting of round cells with homogeneous sizes developed within 1 day, and the numbers of cells increased rapidly (Fig. 1C). When individual day 5 colonies were analyzed, more than 95% of differentiated colonies produced the clusters, whereas less than 5% of undifferentiated colonies did (*10*). If the first passage was not done, hematopoietic cells were buried in the colonies and could not be observed. Cells were usually passed again 5 days after the first passage. This second passage, although not essential for dissecting hematopoiesis, eliminates residual undifferentiated or differentiated colonies. After the second passage, round cells of various sizes predominated

T. Nakano and T. Honjo, Department of Medical Chemistry, Faculty of Medicine, Kyoto University Yoshida, Sakyo-ku, Kyoto, 606, Japan.
H. Kodama, Department of Anatomy, Ohu University School of Dentistry, Tomitacho, Kohriyama, Fukushima, 963, Japan.

... Kodama, M. Nose, S. Niida, S. Nishikawa, S-i. ...nishikawa, *Exp. Hematol.*, in press.
... Yoshida *et al.*, *Nature* **345**, 442 (1990).
The cell line OP9 was maintained in α-modified minimum essential media (α-MEM, Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Whittaker, Walkersville, MD). The same media was used for differentiation induction. The ES cells were maintained with standard procedures, on embryonic fibroblasts with recombinant leukemia inhibitory factor. For differentiation induction, ES cells were seeded onto confluent OP9 cell layers on six-well plates (Nunc, Roskilde, Denmark) at a density of 10^4 cells per well. Both differentiated and undifferentiated cells were trypsinized with 0.25% trypsin for 10 min, suspended with α-MEM supplemented with 20% FCS, and pipetted vigorously until cells became single cells. Then the cells were seeded onto confluent OP9 cell layers on six-well plates at various cell densities.
T. Nakano, unpublished data.
When the cells were transferred at low density, clusters consisting of even two cells did not appear 4 hours after plating, whereas many clusters emerged 1 day later (*10*).
T. Suda, J. Suda, M. Ogawa, J. Ihle, *J. Cell. Physiol.* **124**, 182 (1985).
T. Suda *et al.*, *Blood* **74**, 1936 (1989).
M. Ogawa *et al.*, *EMBO J.* **7**, 1337 (1988); A. Rolink, A. Kudo, H. Karasuyama, Y. Kikuchi, F. Melchers, *ibid.* **10**, 327 (1991); S. Hayashi *et al.*, *J. Exp. Med.* **171**, 1683 (1990).
H. Kodama *et al.*, *J. Exp. Med.* **173**, 269 (1991).
M. R. Capecchi, *Science* **244**, 1288 (1989).
M. L. Mucenski *et al.*, *Cell* **65**, 677 (1991); E. Y. H. P. Lee *et al.*, *Nature* **359**, 288 (1992); T. Jacks *et al.*, *ibid.*, p. 295; A. R. Clarke *et al.*, *ibid.*, p. 328.
J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
A. Shimizu, N. Takahashi, Y. Yaoita, T. Honjo, *Cell* **28**, 499 (1982).
R. Orlandi, D. H. Gussow, P. T. Jones, G. Winter, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3833 (1989); T. Kawakami, N. Takahashi, T. Honjo, *Nucleic Acids Res.* **8**, 3933 (1980).
P. W. Kincade, G. Lee, T. Watanabe, L. Sun, M. P. Scheid, *J. Immunol.* **18**, 97 (1988).
M. Ogawa *et al.*, *J. Exp. Med.* **174**, 63 (1991).
K. Ikuta *et al.*, *Cell* **62**, 863 (1990).
T. A. Springer, G. Galfre, D. S. Secher, C. Milstein, *Eur. J. Immunol.* **8**, 539 (1978).
Preparation of high molecular weight DNA and Southern (DNA) blotting was carried out by standard procedures (*18*). DNA (10 μg) was digested with Eco RI, subjected to electrophoresis in 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled, 0.9-kb Hind III–Xba I JH4 probe (*19*).
RNA was prepared by using TRIzol (Life Technologies, Gaithersburg, MD). About 2 μg of total RNA was used for first strand synthesis by using random hexamer (*18*). The forward primer is VH1BACK, AGGT%A%CA%CTGCAG%AGTC%G. Backward primers are CH1RC, AATGGGCACATGCAGATC-TC; and CH1HE, TCAGACAGGGGGCTCTCG (*20*). The complementary DNA was amplified with VH1BACK primer and CH1RC primer at first, and then the polymerase chain reaction (PCR) product was diluted 1000-fold and amplified with VH1BACK primer and internal primer CH1HE. Samples were amplified for 40 cycles under the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.
We cultured 10^4 D3 ES cells on OP9 cells for 5 days in the presence or absence of M-CSF as indicated. The numbers of differentiated and undifferentiated colonies were counted. Both differentiated and undifferentiated colonies were trypsinized, and 10^5 cells were transferred onto fresh OP9 cells and cultured for another 5 days in the presence or absence of M-CSF as indicated. Ten days after the initiation of the differentiation induction, the number of clusters were counted. After removal of OP9 cells the number of harvested cells were counted, and cytospin specimens stained with May-Grunwald Giemsa were examined to discriminate between macrophages and nonmacrophages. Data in Table 2 are shown as cumulative numbers yielded from 10^4 ES cells at the initiation of the induction.

28. We thank J. Frampton and T. Graf for critical reading of the manuscript, S. Okazaki for excellent technical assistance, K. Yamamura for the D3 ES cell line, T. Kina for TER-119, S. Nishikawa for IL-7 and S-i. Nishikawa and T. Suda for discussions and encouragement.

31 January 1994; accepted 27 June 1994

Delocalization of Vg1 mRNA from the Vegetal Cortex in *Xenopus* Oocytes After Destruction of Xlsirt RNA

Malgorzata Kloc and Laurence D. Etkin*

The Xlsirts are a family of transcribed repeat sequence genes that do not code for protein. Xlsirt RNAs become localized to the vegetal cortex of *Xenopus* oocytes early in oogenesis, before the localization of the messenger RNA Vg1, which encodes a transforming growth factor-β–like molecule involved in mesoderm formation, and coincident with the localization of Xcat2 transcripts, which encode a nanos-like molecule. Destruction of the localized Xlsirts by injection of antisense oligodeoxynucleotides into stage 4 oocytes resulted in the release of Vg1 transcripts but not Xcat2 transcripts from the vegetal cortex. Xlsirt RNAs, which may be a structural component of the vegetal cortex, are a crucial part of a genetic pathway necessary for the proper localization of Vg1 that leads to subsequent normal pattern formation.

Normal development of the vertebrate embryo is dependent on the proper spatial organization of maternally expressed macromolecules in the oocyte. In amphibian oocytes, mRNAs are localized at both the animal and vegetal regions (*1*). The vegetally localized mRNAs include Vg1 (*2*), which encodes a TGF-β–like molecule implicated in mesoderm formation, and Xcat2 (*3*), which encodes a nanos-like molecule. Based on the roles of their putative homologs in *Drosophila*, it is likely that these gene products are involved in axial patterning of the early amphibian embryo. Therefore, unraveling the genetic pathways involved in the localization of these transcripts would give greater insight into how the vertebrate body plan is established.

A group of nontranslatable interspersed repeat transcripts, Xlsirts, is also localized to the vegetal cortex of *Xenopus* oocytes during the early stages of oogenesis and may be a structural component of the cortex involved in the localization of other RNAs (*4*). To determine whether Xlsirts function to localize other RNAs at the vegetal cortex, we injected anti-sense oligodeoxynucleotides (AS ODNs) into stage 4 oocytes (*5*) to destroy the endogenous localized Xlsirt RNAs and analyzed the subsequent distribution of Vg1 and Xcat2 RNAs. Because Xlsirt RNAs do not code for protein, destruction of the RNA with AS ODNs should create a null mutant (*6*, *7*).

The Xlsirt AS ODNs consisted of a mixture of two different phosphothiolated 17-mers. We determined that the optimal dosage was 50 ng [that dose destroyed the Xlsirts without causing nonspecific toxic effects (*8*)]. Xlsirt RNA that was localized at the vegetal cortex was detected in oocytes injected with a control ODN for another maternally transcribed RNA, Xlcaax (formerly Xlgv 7) (*6*, *9*) (Fig. 1A). However, we did not detect any Xlsirt RNA localized at the vegetal cortex by in situ hybridization in oocytes that were injected with Xlsirt AS ODNs and cultured for 3 days (Fig. 1B). Xlcaax ODNs do not produce any nonspecific toxic effects when injected into oocytes (*6*–*8*).

Xlsirts are a heterogeneous population of RNAs consisting of transcripts from both strands of the genes (*4*). Those molecules found localized at the vegetal cortex consist of transcripts from one strand that are referred to as sense strand transcripts. Other Xlsirt RNAs are found throughout the cytoplasm and in the germinal vesicle (GV) and may exist as double-stranded molecules. Xlsirt RNAs appear as a smear on Northern (RNA) blots (*4*, *10*). On Northern blots of RNA isolated from oocytes injected with Xlsirt AS ODNs, we detected a small decrease in the amount of Xlsirt RNA but not the loss of any specific RNA species. This result made it difficult to determine the effi-

Department of Molecular Genetics, University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA.

*To whom correspondence should be addressed.

Fig. 1D), whereas undifferentiated or mesoderm-like colonies were virtually undetectable. After 14 days, round cells began to detach from the stromal cells, presumably because of complete differentiation.

The majority of cells at day 8 looked like immature hematopoietic cells (Fig. 1E). At day 14, 2- to 8 × 10^6 cells arose from 10^4 D3 cells, and more than 90% of the cells exhibited variable morphology of hematopoietic lineage cells (Fig. 1F) consisting of neutrophils, macrophages, erythroid cells, mast cells, megakaryocytes, and lymphoid cells. Differentiation into hematopoietic progenitor cells, erythroid lineage cells, and granulocyte-macrophage cells was confirmed by staining with monoclonal antibodies (mAbs) against c-kit (15%), TER-119 (25%), and Mac-1 (5%), respectively (Fig. 2A). A significant percentage (7%) of the day 14 cells expressed B lineage marker B220 on their surface, although no cells were detected that stained positive for surface immunoglobulin M (IgM). A linear relation was observed between the number of cells plated at day 5 and the clusters formed on OP9 cells 5 days later (Fig. 3), suggesting that the individual clusters are of clonal origin (*11*). The development of mesodermal cells and hematopoietic precursor (or stem) cells from ES cells appeared to occur within the first 5 days, with the proliferation and differentiation of specific hematopoietic lineages occurring later. These sequential processes could be observed under the microscope.

To determine the differentiation capacity of day 10 clusters, we separately picked clusters and transferred them to semisolid culture medium containing interleukin-3 (IL-3) and erythropoietin (Epo) under conditions that promote myeloid cell growth (*12*). Out of 20 clusters, 19 produced colonies consisting of various myeloid lineage cells. More than 75% of the clusters differentiated into mixed colonies (Table 1). Thus, most of the clusters contained hematopoietic progenitor cells that could differentiate along various myeloid lineages.

We also examined the lymphohematopoietic differentiation capacity of individual clusters. Well-separated day 10 clusters were picked, trypsinized, and divided into two aliquots. One aliquot was transferred to semisolid medium under the myeloid condition and the other to semisolid medium containing IL-7 as the growth factor for B lineage cells (*13*). Two of 45 clusters produced colonies under both conditions; the cells that appeared under the myeloid conditions and the B cell conditions consisted of myeloid cells and lymphoid cells, respectively (Fig. 1, G and H). Twenty-three clusters produced only myeloid colonies and 20 did not produce any. The addition of IL-7 and 2-mercaptoethanol (2-ME), which promotes the growth of immature B lineage cells on stromal cells (*14*), caused bursting proliferation of B220-positive and surface IgM–negative immature B lineage cells which completed immunoglobulin DJ gene rearrangement in about 10% of day 14 hematopoietic clusters (Fig. 2, B and D). The lymphoid cells in a small proportion of clusters (one per 500 to 1000 day 14 clusters) persisted and proliferated on OP9 cells after the detachment of the majority of the cells and differentiated in 40 days into surface IgM–positive cells that express the com-

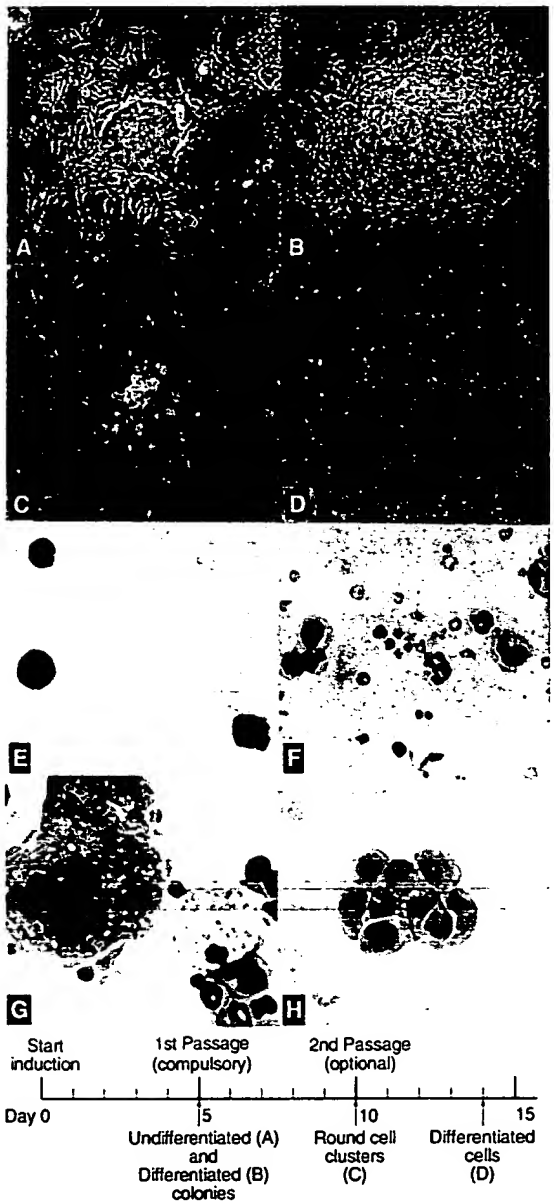


Fig. 1. Photographs of the cells after differentiation induction. Passage of cocultured ES cells was done 5 and 10 days after initiation. The points of phase contrast microscope photographs (**A** to **D**) are indicated at the bottom of the figure: undifferentiated colonies (A), differentiated mesoderm-like colonies (B), hematopoietic cell clusters (C), and differentiated blood cells (D). Cells from clusters (**E**) 8 and (**F**) 12 days after the differentiation induction were prepared by cytocentrifugation (Shandon Southern Products, Sewickly, PA), fixed, and stained with May-Grunwald Giemsa. Day 10 hematopoietic cell clusters were picked up and cultured in semisolid media under (**G**) myeloid conditions or (**H**) B cell conditions. Original magnification, ×100 (A to D) and ×400 (E to H).

Table 1. Differentiation capacity of day 10 clusters in semisolid medium containing IL-3 and Epo. Colonies which appeared 8 days after the transfer of individual day 10 clusters to the semisolid media were picked and their cytospin specimens were stained with May-Grunwald Giemsa. Types of cells are n, neutrophil; m, macrophage; E, erythroid; mast, mast cell; M, megakaryocyte; and blast, blastic cell.

Colony Type						Number
n	m	E	mast	M	blast	2
n	m	E			blast	2
	m	E		M	blast	1
	m	E			blast	1
	m				blast	1
n	m	E	mast			1
	m	E		M		2
	m	E				5
n	m					2
	m					1
		E				1
None						1

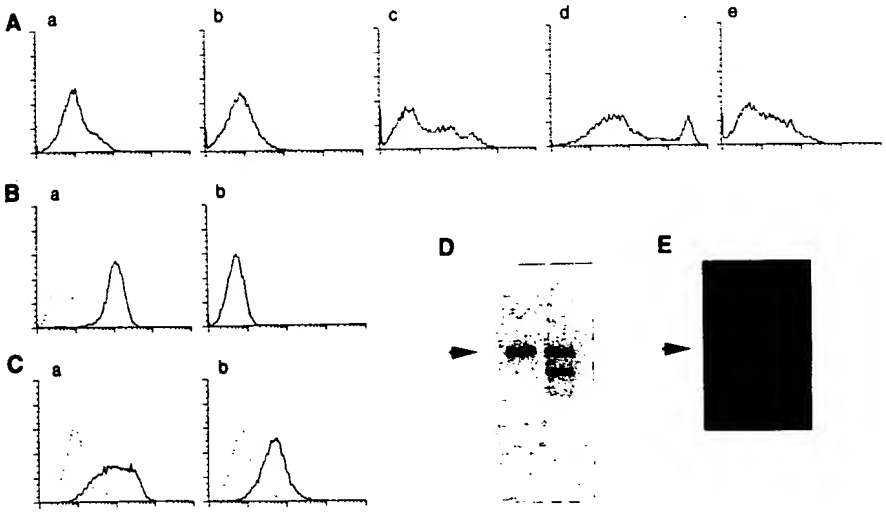


Fig. 2. Expression of lineage markers and immunoglobulin gene rearrangement. (**A** to **C**) Standard two-stage immunofluorescence was performed with optimal concentrations of primary mAb against (a) B220 (hybridoma 6B2) (*21*); (b) IgM [biotinylated goat antibody to mouse IgM $F(ab')_2$]; (c) c-kit, ACK-2 (*22*); (d) an erythroid lineage–specific antigen, TER-119 (*23*); and (e) Mac-1, M1/70.15.1 (*24*). The second stage was fluorescein isothiocyanate (FITC)–conjugated mAb to rat κ (Cosmo, Tokyo, Japan) or FITC-conjugated avidin (Cappel, West Chester, PA). Control samples of B220 were stained with isotype-matched rat IgG antibody and FITC-conjugated mAb to rat κ. The cells were analyzed on a FACScan. Day 14 cells (A) and day 40 cells (C) were maintained on OP9 cells without IL-7 and 2-ME. Day 20 cells (B) were obtained with stimulation of IL-7 and 2-ME after day 10. (**D**) Southern hybridization of DNA of undifferentiated D3 cells (left) and day 20 + IL-7 cells (right) with mouse JH4 probe (*18, 19, 25*). The arrowhead shows the size of the unrearranged JH band. (**E**) Reverse transcriptase PCR analysis of immunoglobulin VDJ transcript of undifferentiated D3 cells (left) and day 40 cells (right) (*20, 26*). The arrowhead shows the expected size of PCR products from VDJ-Cμ transcripts.

Table 2. Effect of M-CSF on the differentiation induction. The values show the cumulative number of clusters and cells yielded from 10^4 D3 ES cells 10 days after transfer to OP9 cells (*27*). Data are mean ± SE of four plates and the *P* value is <0.05 when any combination of data in each column is compared by *t* test. N.C., not countable because of a large number of macrophages diffusely located on dishes.

Addition of M-CSF (day)		Number of small round cell clusters	10^5 cells		
0–5	5–10		Total	Nonmacrophages	Macrophages
–	–	1240 ± 60	17.7 ± 1.6	13.2 ± 0.9	4.5 ± 0.8
–	+	N.C.	78.1 ± 2.3	7.0 ± 1.2	71.1 ± 2.8
+	–	130 ± 10	2.6 ± 0.2	1.9 ± 0.2	0.7 ± 0.1
+	+	N.C.	10.1 ± 0.9	1.0 ± 0.1	9.1 ± 0.8

plete μ chain mRNA (Fig. 2, C and E).

We confirmed the inhibitory effect of M-CSF on the differentiation by adding recombinant human M-CSF (200 ng/ml) (*15*). Addition of M-CSF during the first 5 days reduced the number of differentiated colonies to about one-third but had no influence on the formation of undifferentiated colonies (*10*). This indicates that M-CSF inhibits differentiation of ES cells into mesodermal cells. When M-CSF was added for the first 5 days of the 10-day culture, the number of day 10 hematopoieitc clusters was reduced to about 10%, and those of total cells and nonmacrophages to one-seventh irrespective of the presence of M-CSF during the second 5 days (Table 2). Thus, M-CSF inhibits not only differentiation of ES cells into mesodermal cells but also subsequent development to hematopoietic cells. When M-CSF was added during the second 5 days, hematopoietic cell clusters were hardly detectable, instead there was a tremendous proliferation of macrophages. Although five times more cells appeared, 90% of the cells were macrophages, and the number of nonmacrophages was reduced to about half. This increase of macrophages and decrease of nonmacrophages occurred regardless of the addition of M-CSF during the first 5 days. M-CSF unexpectedly inhibited the differentiation from ES cells to hematopoietic cells at more than one step. This explains why effective differentiation from ES cells to blood cells took place only on the M-CSF–deficient stromal cell line.

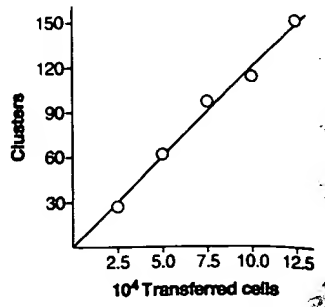


Fig. 3. Relation between numbers of transferred cells obtained by coculturing for 5 days on OP9 cells and those of hematopoietic clusters after culturing for another 5 days.

The other in vitro differentiation induction systems rely on the formation of embryoid bodies, suggesting the involvement of complex induction mechanisms for the development of blood cells (*1, 2*). Differentiated mesodermal colonies in our system are different from embryoid bodies because (i) differentiated colonies grew flat and did not pile up until day 3 or 4, (ii) the colonies did not form complex structures such as cysts or lumina even after piling up, and (iii) erythroid and multipotential precursors appeared early (by day 3 and day 4, respectively) than in the embryoid body–forming systems (*2, 10*). These observations suggest that direct interactions with an M-CSF–deficient stromal cell line may be sufficient to induce the development of lymphohematopoietic cells, although requirement of some interactions between the mesodermal cells during the first 5 days could not be excluded. Eighteen out of 30 randomly chosen G418-resistant ES cell clones differentiated into hematopoietic cells as efficiently as parental ES cells (*10*). This system will thus allow the study of hematopoietic cell development with in vitro genetic manipulation of ES cells (*16*) while avoiding the generation of lethal phenotypes that the targeted disruption of developmental genes often causes (*17*).

REFERENCES AND NOTES

1. T. C. Doetschman, H. Eistetter, M. Katz, J. Schmidt, R. Kemler, *J. Embryol. Exp. Morphol.* **87**, 27 (1985).
2. M. V. Wiles and G. Keller, *Development* **111**, 259 (1991); R. M. Schmitt, E. Bruyns, R. Snodgrass, *Genes Dev.* **5**, 728 (1991); U. Burkert, T. Ruden, E. F. Wagner, *New Biol.* **3**, 698 (1991); U. Chen, M. Kosco, U. Staerz, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2541 (1992); U. Chen, *Dev. Immunol.* **2**, 29 (1992); G. Keller, M. Kennedy, T. Papayannopoulou, M. A. Wiles, *Mol. Cell. Biol.* **13**, 473 (1993).
3. J. Gutierrez-Ramos and R. Palacios, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9171 (1992).
4. H. Kodama, H. Sudo, H. Koyama, S. Kasai, S. Yamamoto, *J. Cell. Physiol.* **118**, 233 (1984); T. Sudo *et al.*, *J. Exp. Med.* **170**, 333 (1989).
5. G. V. Borzillo, R. A. Ashmun, C. J. Sherr, *Mol. Cell. Biol.* **10**, 2703 (1990).

RBI-J5

STIC-ILL

1632

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic

Gen tically Selected Cardiomyocytes from Differentiating Embryonic Stem C lls Form Stabl Intracardiac Grafts

Michael G. Klug, Mark H. S onpaa, Gou Y ung K h, and Loren J. Field
Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202-4800

Abstract

This study describes a simple approach to generate relatively pure cultures of cardiomyocytes from differentiating murine embryonic stem (ES) cells. A fusion gene consisting of the α-cardiac myosin heavy chain promoter and a cDNA encoding aminoglycoside phosphotransferase was stably transfected into pluripotent ES cells. The resulting cell lines were differentiated in vitro and subjected to G418 selection. Immunocytological and ultrastructural analyses demonstrated that the selected cardiomyocyte cultures (> 99% pure) were highly differentiated. G418 selected cardiomyocytes were tested for their ability to form grafts in the hearts of adult dystrophic mice. The fate of the engrafted cells was monitored by antidystrophin immunohistology, as well as by PCR analysis with primers specific for the myosin heavy chain–aminoglycoside phosphotransferase transgene. Both analyses revealed the presence of ES-derived cardiomyocyte grafts for as long as 7 wk after implantation, the latest time point analyzed. These studies indicate that a simple genetic manipulation can be used to select essentially pure cultures of cardiomyocytes from differentiating ES cells. Moreover, the resulting cardiomyocytes are suitable for the formation of intracardiac grafts. This selection approach should be applicable to all ES-derived cell lineages. (*J. Clin. Invest.* 1996. 98:216–224.) Key words: myocardial repair • infarct • somatic cell therapy

Introduction

Cardiomyocyte loss in the adult mammalian heart is irreversible and frequently leads to diminished cardiac function. The ability to increase the number of functional cardiomyocytes in a diseased heart would have obvious therapeutic potential. Recent studies have suggested that cellular engraftment can be used to augment myocyte number in the adult heart. Although several different types of myocyte preparations have been successfully engrafted (1–8), the inherent electrophysiologic, structural, and contractile properties of cardiomyocytes strongly suggest that they are the ideal donor cell type. However, difficulties in obtaining sufficient numbers of fetal donor cells might constitute a serious impediment for the widespread implementation of intracardiac engraftment. Identification of an alternative source of donor cardiomyocytes would thus be of value.

Embryonic stem (ES)[1] cells, totipotent cell lines derived from the inner cell mass of blastocysts, have the ability to differentiate into a variety of cell lineages in vitro (9). Cardiogenic induction during ES differentiation is manifest by the appearance of spontaneously and rhythmically contracting myocytes. ES-derived cardiomyocytes express α- and β-cardiac myosin heavy chain (MHC) (10), α-tropomyosin (11), myosin light chain 2v (MLC-2v) and atrial natriuretic factor (12), phospholamban (13) and type B natriuretic factor (14), and exhibit normal contractile sensitivity to calcium (15). Electrophysiologic analyses have identified action potentials typical for atrial, ventricular, and conduction system cardiomyocytes from ES-isolated cell preparations (16, 17). Furthermore, cell cycle withdrawal and multinucleation in ES-derived cardiomyocytes follow a temporal program roughly similar to that observed during cardiogenesis in vivo (18). These attributes suggested that differentiating ES cells might constitute a renewable source of donor cardiomyocytes suitable for cardiac engraftment.

However, successful use of ES-derived donor cells would require the generation of essentially pure cardiomyocyte cultures, as engraftment of pluripotent or totipotent ES cells would likely result in teratoma formation. In this study, totipotent ES cells were genetically modified so as to permit cardiomyocyte enrichment. ES cell lines carrying a fusion gene comprised of the α-cardiac MHC promoter and sequences encoding aminoglycoside phosphotransferase were generated. Expression of the fusion gene in ES-derived cardiomyocytes facilitated their selection with G418 after in vitro differentiation. Immunohistologic and ultrastructural analyses revealed that the selected cardiomyocytes were highly differentiated. The G418 selected cardiomyocytes were tested for their ability to form grafts in the hearts of adult mice. Stable ES-derived cardiomyocyte grafts were observed as long as 7 wk after implantation. Thus, a relatively simple scheme of genetic selection can be used to generate pure cultures of differentiated cardiomyocytes. Moreover, the selected cells were able to form stable intracardiac grafts. This selection approach should be applicable to all ES-derived cell lineages.

Methods

Generation of the myosin heavy chain–aminoglycoside phosphotransferase transgene. A molecule carrying both an α-cardiac myosin heavy chain–aminoglycoside phosphotransferase (MHC-neo^r) and a phosphoglycerate kinase (pGK)-hygromycin resistant transgene in a common pBM20 vector backbone (Boehringer Mannheim, Indianapolis,

Address correspondence to Loren J. Field, Krannert Institute of Cardiology, 1111 West 10th Street, Indianapolis, IN 46202-4800. Phone: 317-630-7776; FAX: 317-274-9697. Gou Young Koh's present address is Department of Physiology, Chonbuk Medical School, San 2-20, Keum-Am-Dong, Chonju 560-182, South Korea.

Received for publication 4 December 1995 and accepted in revised form 12 April 1996.

J. Clin. Invest.

0021-9738/96/07/216/09 $2.00
Volume 98, Number 1, July 1996, 216–224

1. *Abbreviations used in this paper:* ES, embryonic stem; LIF, leukemia inhibitory factor; MHC, myosin heavy chain; MLC, myosin light chain; neo^r, neomycin resistance; pGK, phosphoglycerate kinase.

IN) was generated. The α-cardiac MHC promoter consisted of 4.5 kb of 5′ flanking sequence and 1 kb of the gene encompassing exons 1 through 3 up to but not including the initiation codon (19). The aminoglycoside phosphotransferase (neo^r) cDNA was subcloned from pMC1-neo poly A (Stratagene, La Jolla, CA). The pGK-hygromycin sequences were described previously (20). The transgene is depicted schematically in Fig. 1. Transgene insert (containing both the MHC-neo^r and pGK-hygromycin sequences) was isolated by digestion with XhoI and HinDIII and transfected into ES-D3 stem cells (American Type Culture Collection, Rockville, MD) via electroporation. Transfected clones were selected by growth in the presence of hygromycin (200 μg/ml; Calbiochem-Novabiochem, La Jolla, CA). PCR analyses were used to confirm that both the MHC-neo^r and pGK-hygromycin sequences were present in the resulting cell lines. The ES cells were maintained in the undifferentiated state by culturing in high glucose DME containing 10% FBS, nonessential amino acids, and 0.1 mM 2-mercaptoethanol. The medium was supplemented to a final concentration of 100 U/ml with conditioned medium containing recombinant leukemia inhibitory factor (LIF) as described (21).

In vitro differentiation and selection of ES-derived cardiomyocytes. To induce differentiation, 2×10^6 freshly dissociated ES cells were plated onto a 100-mm bacterial Petri dish containing 10 ml of DME lacking supplemental LIF. After 3 d in suspension culture, the resulting embryoid bodies were plated onto plastic 100-mm cell culture dishes and allowed to attach. Regions of cardiogenesis were readily identified by the presence of spontaneous contractile activity. For cardiomyocyte selection, the differentiated cultures were grown for 8 d in the presence of G418 (200 μg/ml; Gibco Laboratories, Grand Island, NY).

To determine the cardiomyocyte content in nonselected cultures, plates were treated with trypsin 16 d after cardiogenic induction, and the resulting suspension was plated at low density onto fibronectin-coated cell culture dishes to permit visualization of individual cells. After 24 h of culture, the cells were fixed in acetone and processed for immunocytology with MF20, an anti-MHC antibody (see below). To determine the cardiomyocyte content following physical enrichment protocols, rhythmically contracting regions were microdissected with a sterile Pasteur pipette 16 d after cardiogenic induction, dissociated with trypsin, and processed for MHC immunoreactivity. To determine the cardiomyocyte content in selected cultures, ES cells were allowed to differentiate for 8 d following cardiogenic induction. The cultures were then subjected to G418 selection for an additional 8 d. The selected cells were dissociated and processed for MHC immunoreactivity.

Immunofluorescence analyses of cultured ES-derived cardiomyocytes. Cultures of selected EA3 cardiomyocytes were treated with trypsin, and the resulting suspension was cultured at low density onto fibronectin-coated cell culture dishes for 24 h to permit visualization of individual cells. The cultures were then fixed in acetone, and nonspecific immunoreactivity was blocked by incubation in 1% BSA, 10% goat serum, 1× PBS. The primary antibodies used were: MF20 (antisarcomeric myosin), 9D10 (antititin), EA-53 (anti-α-actinin; Sigma Immunochemicals, St. Louis, MO), D3 (antidesmin), and 6-10 (antidystrophin). Secondary antibody was fluorescein- or rhodamine-conjugated anti-mouse or anti-rabbit IgG F(ab′)$_2$ fragment (Boehringer Mannheim). All samples were counterstained with Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) and visualized by epifluorescence.

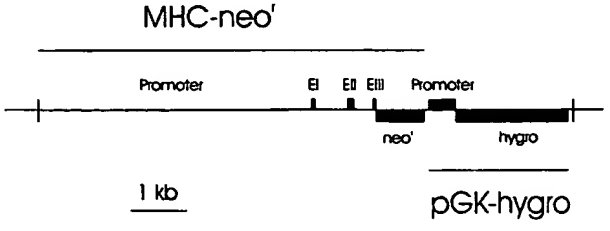


Figure 1. Structure of the MHC-neo^r/pGK-hygromycin transgene. The plasmid backbone was pBM20.

Ultrastructural analysis of selected ES-derived cardiomyocytes. Selected cell cultures were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.1 M sucrose and 0.05% $caCl_2$, followed by postfixation in 2% osmium tetroxide in the same buffer (Stevens Metallurgical Corp., New York). All other chemicals were obtained from Ladd Research Industries, Inc. (Burlington, VT). Samples were stained with 2% uranyl acetate in pH 5.2 maleate buffer (0.05 M), dehydrated, and embedded in Ladd LX-112. After trimming, the block was thin sectioned and stained with uranyl acetate and lead citrate. Specimens were viewed on a Phillips 400 transmission electron microscope.

Myocardial engraftment of selected ES-derived cardiomyocytes. Cultures of selected ES-derived cardiomyocytes were digested with trypsin, and the resulting single cell preparation was washed three times with 1× DME and directly injected into the ventricular myocardium of anesthetized, intubated adult mdx mice (The Jackson Laboratory, Bar Harbor, ME) using a 30-gauge tuberculin syringe as described previously (8). After extubation and evacuation of the pneumothorax, the animals were placed at 37°C and monitored until they recovered from surgery. All animal procedures were in accordance with institutional and National Institutes of Health guidelines.

Immunohistology of hearts. Hearts were harvested, cryoprotected in 30% sucrose, embedded and sectioned at 10 μm on a cryomicrotome as described previously (7, 8). These studies used fetal mice (embryonic day 15 C3HeB/FeJ), normal adult mice (C3HeB/FeJ), and dystrophic adult mice (C57Bl/10ScSn-mdx/J). All animals were obtained from The Jackson Laboratory. Nonspecific immunoreactivity was blocked by incubation in 1% BSA, 10% goat serum, 1× PBS. For antidystrophin immunohistology, sections were incubated with 6-10 (gift of Dr. T. Byers, Indiana University) or DYS1 (Novocastra Laboratories, Newcastle-upon-Tyne, United Kingdom) in 1× PBS, 1% BSA for 2 h at 25°C, rinsed and reacted with a fluorescein-conjugated secondary antibody.

Northern blot analyses. Tissue or cells were homogenized in 4.0 M guanidinium thiocyanate and RNA purified by centrifugation through 5.7 M CsCl using standard protocols as described (22). RNA samples were quantitated by spectrophotometry at 260 nm. For Northern analysis, RNA was denatured with glyoxal, separated by size on 1.2% agarose gels, and transferred to Genescreen (DuPont, Wilmington, DE). Oligonucleotide probes were labeled using polynucleotide kinase under standard conditions (22). Hybridizations were for 20 h at 65°C in 4× SSC, 2× Denhardt's, 0.1% SDS, and 1 mg/ml salmon sperm DNA. Blots were washed at 60°C in 2× SSC, 0.1% SDS, and signal was visualized by autoradiography at −70°C with an intensifying screen. The MLC-2v probe was 5′-CACAGCCCTGGGATGGAGAGTGGGCTGTGGGTCACCTGAGGCTGTGGTTCAG-3′; the MLC-2a probe was 5′-GAGGTGACCTCAGCCTGTCTACTCCTCTTTCTCATCCCCG-3′. Quality of the RNA samples was confirmed by Northern analysis with a murine 18s rRNA oligonucleotide probe (5′-TCCATTATTCCTAGTGCGGTATCCAGGAGGATCGGGCCTGCTTT-3′).

Southern blot analyses. DNA from EA3 cells or from sections of engrafted hearts was prepared by digestion in a solution containing proteinase K (0.1 μg/ml), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 10 mM DTT, 0.005% SDS. The samples were digested at 55°C for 2–4 h. The proteinase K was heat inactivated and the resulting crude nucleic acid preparation was amplified by PCR as described previously (23). The sense primer (5′-CTCTGACAGAGAAGCAGGCACTTTACATGG-3′) was located in the α-MHC promoter and the antisense primer (5′-ATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCA-3′) was located in the neo^r cDNA. The amplification products were transferred to Genescreen Plus (DuPont) and hybridized with a nick-translated probe using standard conditions (22). The hybridization probe encompassed sequences of the MHC-neo^r trans-

gene internal to the oligonucleotide primers used for PCR amplification.

Results

The structure of the MHC-neo'/pGK-hygromycin transgene is depicted in Fig. 1. Initial transfection experiments produced five hygromycin-resistant ES cell lines. PCR analyses confirmed that the cell lines carried both the MHC-neo' and the pGK-hygromycin fusion genes (Klug, M.G., unpublished observation). One line, designated EA3, was chosen for subsequent analyses. To induce differentiation, EA3 cells were plated in suspension culture in the absence of supplementary LIF. 3 d later, the resulting embryoid bodies were replated

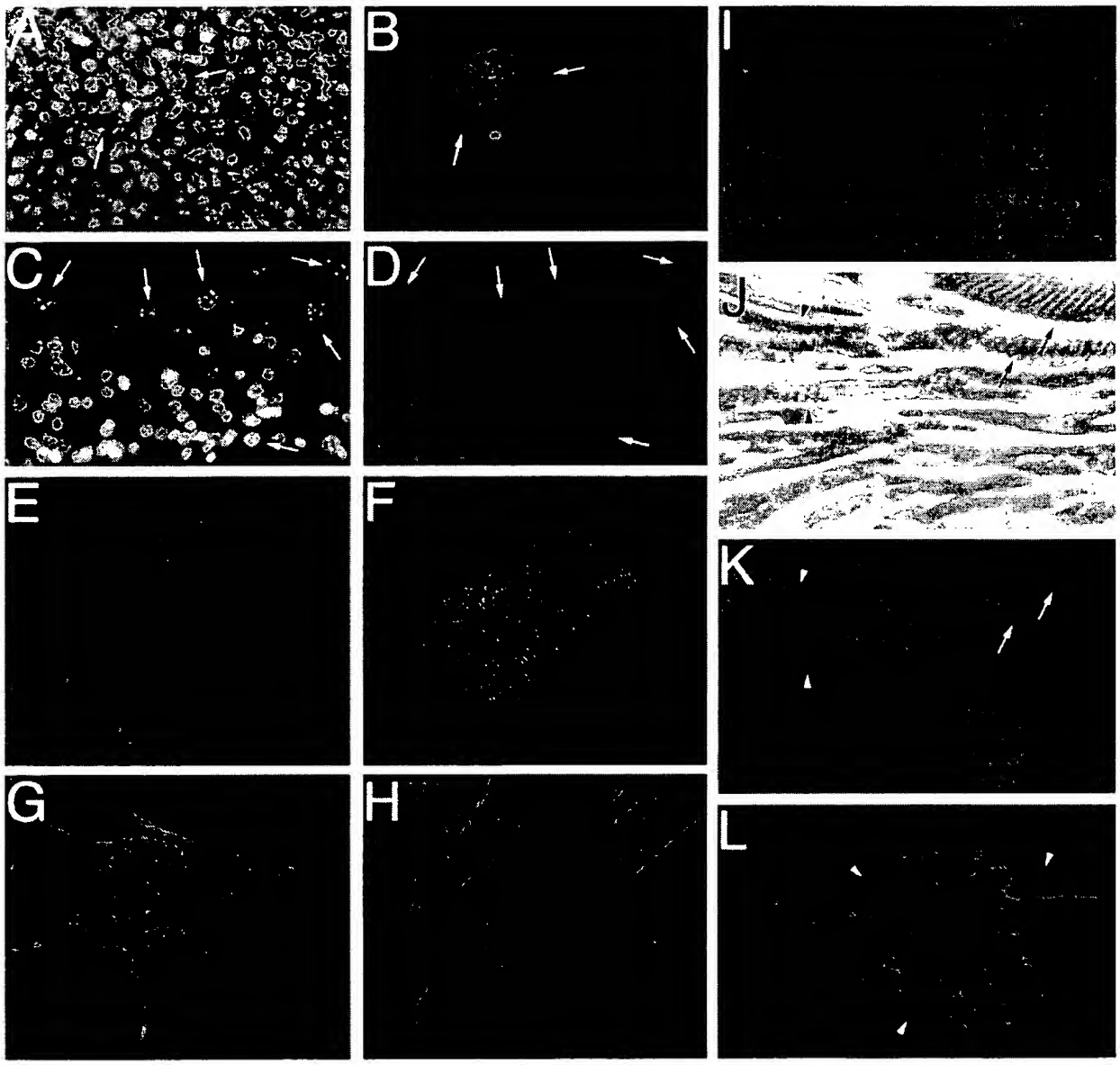


Figure 2. G418 selection of ES-derived cardiomyocytes in vitro and formation of intracardiac grafts. (*A*) Hoechst epifluorescence of a nonselected culture of EA3 ES cells 16 d after differentiation was induced. Note the high density of cells present in the field. (*B*) Antisarcomeric MHC immunofluorescence (green signal) of the same field depicted in *A*. Note that only a few cells are cardiomyocytes (arrows demarcate the same group of cells in *A* and *B*). (*C*) Hoechst epifluorescence of a G418-selected culture of EA3 ES cells 16 d after cardiogenic induction. (*D*) Antisarcomeric myosin immunofluorescence (green signal) of the same field depicted in *C*. Note that all of the cells presented in the G418-selected culture express sarcomeric myosin (arrows demarcate the same group of cells in *C* and *D*). (*E–I*) Immunocytologic analysis for titin, α-actinin, desmin, sarcomeric myosin, and dystrophin expression, respectively, in G418-selected EA3 cardiomyocytes. (*J* and *K*) Phase-contrast image and antidystrophin immunofluorescence, respectively, of an mdx heart engrafted with G418-selected cardiomyocytes. The same field is depicted in *J* and *K*, and dystrophin immunoreactivity appears as green signal in *K*. Arrows indicate myofiber-containing host cells (dystrophin negative), while arrowheads indicate myofiber-containing donor cells (dystrophin positive). (*L*) Antidystrophin immunofluorescence of a second G418-selected cardiomyocyte graft. Dystrophin immunoreactivity appears as green signal.

Table I. Relative Cardiomyocyte Content in Nonselected, Physically Selected, and G418 Selected Cultures of Differentiating EA3 ES Cells

Preparation	Sarcomeric myosin positive cells	Sarcomeric myosin negative cells	Percent cardiomyocytes
No selection*	11	2000	0.55
Physical isolation‡	68	2000	3.4
G418 selection§	791	3	99.6

Analyses were performed on multiple dishes obtained from the same differentiating culture to control for variation in cardiogenic induction. *16 d after cardiogenic induction, plates were treated with trypsin and the resulting suspension was plated at low density onto fibronectin-coated cell culture dishes. After 24 h of culture, sarcomeric myosin immunoreactivity was scored (see Methods). ‡16 d after cardiogenic induction, rhythmically contracting regions were microdissected with a sterile Pasteur pipette, dissociated with trypsin, and the resulting suspension was plated at low density onto fibronectin-coated cell culture dishes. After 24 h of culture, sarcomeric myosin immunoreactivity was scored. §8 d after cardiogenic induction, cultures were treated with G418. After culturing in the presence of G418 for a total of 8 additional d, the selected cells were dissociated with trypsin and the resulting suspension was plated at low density onto fibronectin-coated cell culture dishes. After 24 h of culture, sarcomeric myosin immunoreactivity was scored.

onto tissue cultures dishes and allowed to form adherent colonies. Hoechst epifluorescence analyses demonstrated a very high cellular density in these cultures after a total of 16 d of in vitro differentiation. (Fig. 2 *A*). Immunofluorescence analysis of the same field revealed that only a small percentage of the differentiated cells expressed sarcomeric myosin, indicating that the vast majority of the cells were not cardiomyocytes (Fig. 2 *B*). To quantitate the cardiomyocyte content, the differentiated cultures were digested with trypsin and replated at a lower density to assess the immunoreactivity of individual cells. These analyses confirmed that cardiomyocytes (i.e., MF20 immunoreactive cells) comprised < 1% of the cultured cells (Table I).

To determine the extent of cardiomyocyte enrichment obtained by simple physical isolation, EA3 cells were differentiated in vitro for 16 d using the same procedure described above. Regions exhibiting spontaneous contractile activity were microdissected, digested with trypsin, and replated at a density suitable for immunocytologic analysis of individual cells. Although an ~ 10-fold enrichment of cardiomyocytes was observed, cardiomyocytes comprised only 3.4% of the cells present in microdissected cultures (Table I). To determine the extent of cardiomyocyte enrichment obtained with genetic selection, EA3 cells were differentiated using the same approach described above. After 8 d of differentiation, G418 selection was imposed and the cells were cultured for an additional 8 d. Hoechst epifluorescence analyses indicated that the cellular density in these cultures was markedly reduced as compared with similarly aged nonselected EA3 cultures (Fig. 2, *C* and *A*, respectively). Immunofluorescence analysis of the same field of G418-selected cells indicated that the vast majority of the surviving cells exhibited sarcomeric myosin immunoreactivity (Fig. 2 *D*). Immunofluorescence analysis of single cell preparations was once again used to quantitate the extent of cardiomyocyte enrichment. More than 99% of the cells in G418-selected cultures expressed sarcomeric myosin (Table I). Thus, in the absence of enrichment, only a few cells in differentiated cultures were cardiomyocytes. In contrast, the G418 selection of differentiating EA3 cells produced essentially pure cultures of cardiomyocytes.

Additional studies were performed to characterize the G418-selected cells. Previous studies have shown that the presence of sarcomeric myosin immunoreactivity coupled with the absence of nebulin immunoreactivity permitted the definitive identification of cardiomyocytes in differentiating ES cultures (18). As expected, the G418-selected cells exhibited sarcomeric myosin immunoreactivity but not nebulin immunoreactivity, confirming that the selected cultures were comprised of cardiomyocytes (Table II). The extent of differentiation in G418-selected cardiomyocyte cultures was further assessed by immunocytologic analyses. Once again, the selected cultures were digested with trypsin and replated at a density sufficient for the analysis of individual cells. Well-developed myofibrillar structure was detected by titin and α-actinin immunocytology (Fig. 2, *E* and *F*, respectively). Intermediate filament structure typical of cardiomyocytes was detected by desmin immunocytology (Fig. 2 *G*). High power views of the MHC immunoreactivity revealed the presence of well-aligned myofibers in adjacent G418-selected cells (Fig. 2 *H*). Dystrophin immunoreactivity was also detected in the selected cardiomyocyte cultures (Fig. 2 *I*); the dystrophin gene product is a well-established marker for both cardiac and skeletal muscle. The immunocytological analyses of the selected EA3 cultures are summarized in Table II. In each instance, cardiac tissue from normal fetal, normal adult, and dystrophic adult mice were included as controls.

Heterogeneous ANF immunoreactivity was observed in G418-selected EA3 cultures (Table II). It is well-established that ANF is expressed constitutively in the atrium during development. Although ANF is also expressed at high levels in the fetal ventricle, both transcription and protein accumulation at this site are dramatically downregulated at birth (24, see also Table II). Thus, the heterogeneous ANF expression observed in selected EA3 cultures suggests that these cells have differentiated into the adult phenotype, and that cells with attributes similar to either atrial or ventricular cardiomyocytes are present.

To further address this issue, Northern blots were performed with RNA prepared from adult atria, adult ventricles, undifferentiated EA3 cells, and G418-selected EA3 cardiomyocyte cultures. The blots were hybridized with oligonucleotide probes from the 3′ untranslated region of the MLC-2v and MLC-2a genes (Fig. 3). The hybridization signals observed with the atrial and ventricular RNA samples established the relative specificity of the probes. No signal was observed with RNA prepared from undifferentiated EA3 cultures. In contrast, both the MLC-2v and MLC-2a probes hybridized to RNA prepared from selected EA3 cultures, consistent with the notion that both atrial- and ventricular-like cardiomyocytes can be selected by this process. The relatively low MLC-2v and MLC-2a signal observed in the selected cardiomyocyte cultures as compared with the dissected tissue samples most likely reflects the fact that the cultures are a mixture of atrial- and ventricular-like cardiomyocytes where the tissue samples were not. The potential contribution of conduction cells to the selected cultures would also impact on the relative MLC signals. In addition, the cultured cells were not under mechanical load, and as such MLC-2 transcription may not have been

Table II. Comparative Immunoreactivity of Murine Cardiac Muscle and G418-selected Cardiomyocyte Preparations

Marker*	Cell type			
	Normal embryonic cardiac muscle	Normal adult cardiac muscle	Dystrophic adult cardiac muscle	G418-selected cardiomyocytes
Myosin heavy chain (MF20)	+‡	+	+	+
Titin (9D10)	+	+	+	+
α-Actinin (EA-53)	+	+	+	+
Desmin (D3)	+	+	+	+
Nebulin (NB2)	−	−	−	−
ANF (MA-APIII)	+	±§	±§	±§
Dystrophin (6–10)	±	+	−	+
Dystrophin (DYS1)	±	+	−	+
Utrophin (DRP2)	±‖	−	+	±‖

*EA-53 and NB2 were from Sigma Immunochemicals, DYS1 and DRP2 were from Novocastra Laboratories. Sources for the other antibodies are listed in the Methods and Acknowledgments section. ‡ + indicates strong signal; ± indicates heterogenous signal; − indicates no signal. §ANF was detected in the atria but not ventricles of all adult histologic sections. Only a small percentage of the G418-selected cardiomyocytes exhibited ANF immunoreactivity. ‖Utrophin immunoreactivity was heterogeneous in normal embryonic cardiac muscle and in G418-selected cardiomyocytes.

maximally induced. Finally, the RNA input in the selected cardiomyocyte lanes was low compared with the tissue controls (compare the relative 18s rRNA signals).

The G418-selected cardiomyocytes exhibited spontaneous and rhythmic contractile activity, with adjacent cells beating synchronously. Cultures of selected, beating cardiomyocytes could be maintained for as long as 11 mo (Klug, M.G., unpublished observation). Ultrastructural analyses further established the differentiated status of EA3 cardiomyocyte cultures (Fig. 4). Normal adult sarcomeric structure was apparent in the selected cells. Intercalated discs composed of fascia adherens, desmosomes, and gap junctions were observed to connect adjacent cells (Fig. 4 *B*). The laminar structure of the gap junctions was confirmed by examination at high magnification (Fig. 4 *C*). Myosin synthesis was observed to occur along free ribosomes in many of the selected cells (Fig. 4 *D*). Collectively, these light and electron microscopic analyses indicated that G418 selection did not impact negatively upon cardiomyocyte differentiation.

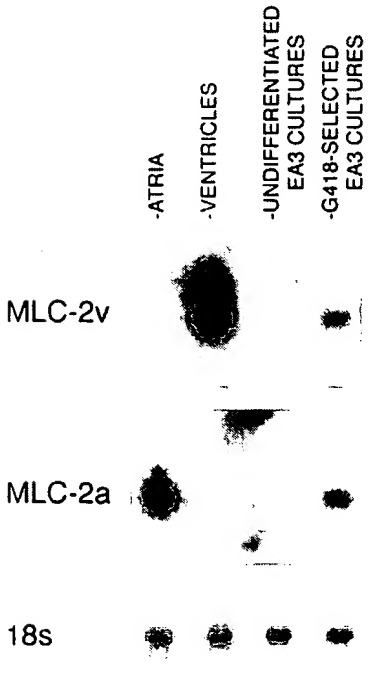


Figure 3. MLC-2a and MLC-2v are expressed in G418-selected EA3 cultures. RNA from adult atria, adult ventricles, undifferentiated EA3 cells, or G418-selected EA3 cultures was hybridized to oligonucleotide probes from the MLC-2v (*top*) or MLC-2a (*middle*) genes. The integrity and quantity of the RNA samples was assessed by hybridization with an 18s rRNA probe (*bottom*).

Selected cardiomyocytes were tested for their ability to generate intracardiac grafts. G418-treated EA3 cultures were digested with trypsin, and $\sim 1 \times 10^4$ cells were delivered into the left ventricular free wall of dystrophic mdx recipient mice. Given that the selected cardiomyocytes expressed dystrophin (see Fig. 2 *I* and Table II) whereas the myocardium of the recipient mdx mice did not (see Table II, also reference 25), the fate of engrafted donor cells was readily monitored by antidystrophin immunohistology. Phase-contrast microscopic examination of cryosections of the recipient hearts revealed that engrafted regions frequently exhibited normal myocardial topography (Fig. 2 *J*). Immunocytologic assays with antidystrophin antibody 6-10 revealed the presence of dystrophin-positive G418-selected cardiomyocytes (Fig. 2 *K* and *L*). Comparison of phase-contrast and antidystrophin images of the same field revealed the presence of myofibers in the engrafted G418-selected cardiomyocytes (Fig. 2, *J* and *K*, respectively).

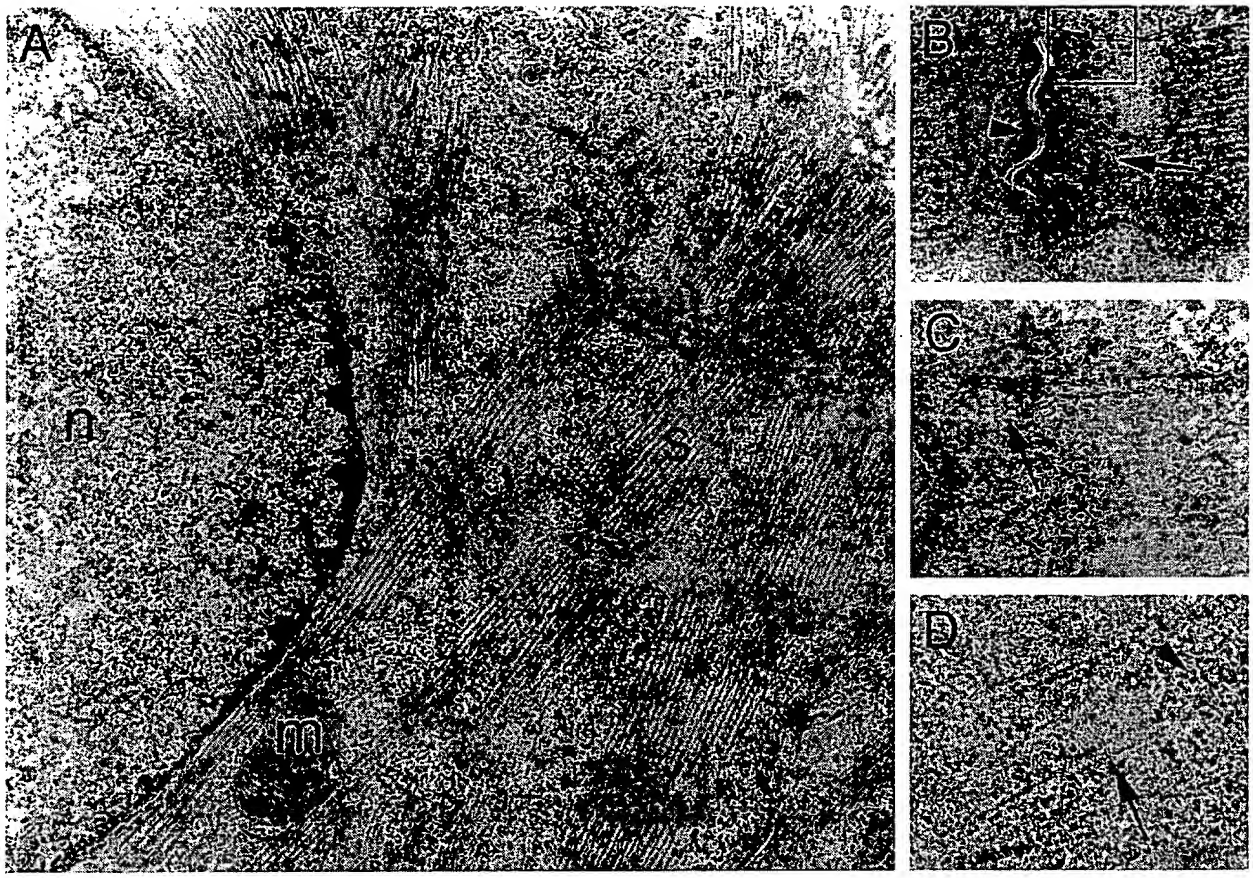


Figure 4. Ultrastructural analysis of selected EA3 cardiomyocytes. (*A*) Low-power transmission electron micrograph of a G418-selected cardiomyocyte: *n*, nucleus; *m*, mitochondria; *s*, sarcomere. (*B*) High-power electron micrograph showing the presence of a desmosome (*arrowhead*), fascia adherens (*arrow*), and gap junctions (*boxed*), which are the ultrastructural components of intercalated discs. (*C*) High-power view of the boxed region in *B* demonstrating the laminar structure of the gap junctions. (*D*) High-power electron micrograph demonstrating the presence of myosin synthesis (*arrows*) along free ribosomes (*arrowheads*).

6-10, a relatively high-affinity polyclonal antibody, recognizes the rod domain of dystrophin (amino acid residues 1991–3112). To confirm that this immunoreactivity was not artifactual (i.e., an "immunologic revertant," see reference 25), adjacent sections of the engrafted hearts were examined with an anti-dystrophin monoclonal antibody (DYS1) which recognizes a different epitope (amino acid residues 1181–1388). 83% of the 6-10 positive grafts exhibited DYS1 immunoreactivity on adjacent sections (Table III). Importantly, no DYS1 immunoreactivity was observed in multiple sections of control mdx samples. Given the unequivocal nature of the DYS1 control experiment, the positive signal observed in these hearts strongly suggests that the G418-selected cardiomyocytes were engrafted successfully. Six of the eight animals analyzed developed grafts exhibiting both 6-10 and DYS1 immunoreactivity; a similar success rate was obtained with fetal cardiomyocyte grafts (7, 8).

Previous studies with a canine dystrophic model used utrophin immunohistology to provide a second, albeit negative, marker for engrafted cardiomyocytes (8). Although utrophin-negative, dystrophin-positive cardiomyocytes were detected in the engrafted hearts in this study, the heterogeneous pattern of utrophin expression observed in cultured EA3 cardiomyocytes precluded the use of this assay as second marker for engrafted cells. Therefore PCR amplification with primers specific for the MHC-neo^r fusion gene was used to confirm the presence of engrafted G418-selected cardiomyocytes. DNA was prepared

Table III. Summary of Dystrophin Immunoreactivity and PCR Analyses of mdx Hearts Engrafted with G418-selected Cardiomyocytes

Mouse	Dystrophin (6–10)	Dystrophin (DYS1)	PCR
1	+*	+	–
2	+	–	ND‡
3	–	–	ND
4	+	+	+
5	–	–	ND
6	+	+	+
7	+	+	+
8	+	+	+

*+ indicates signal detected; – indicates no signal detected; ND indicates not determined. ‡PCR analyses were performed only on those grafts which exhibited both 6–10 and DYS1 immunoreactivity.

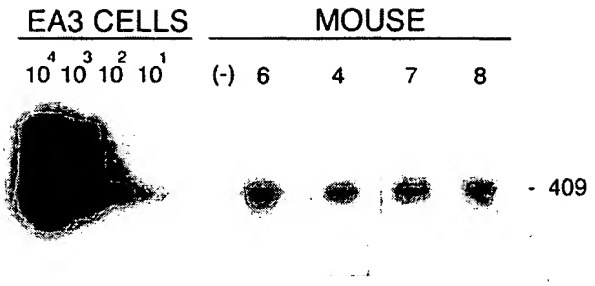


Figure 5. Southern blot analysis of PCR-amplified DNA prepared from mdx hearts engrafted with G418-selected ES-derived cardiomyocytes. DNA from 10^4, 10^3, 10^2, or 10^1 EA3 cells, from a nonengrafted heart section (−), or from engrafted heart sections exhibiting DYS1 immunoreactivity was amplified with primers specific for the MHC-neo^r transgene (mouse number refers to the designations presented in Table III). The resulting amplification products were separated on agarose gels, transferred to nylon membrane, and hybridized with a nick-translated probe corresponding to sequences of the transgene internal to the primers used for PCR amplification. The 409-bp MHC-neo^r transgene amplification product was readily detected in the samples prepared from DYS1-positive engrafted hearts, as well as in the positive control samples from EA3 cells. No signal was detected in the amplification products generated from nonengrafted heart sections.

from engrafted heart sections exhibiting DYS1 immunoreactivity, as well as from sections of nonengrafted control hearts. DNA from the parental EA3 cell line was used as a positive control. The DNA samples were subjected to PCR amplification, and the products were separated on agarose gels, transferred to nylon membranes, and hybridized with a nick-translated probe which encompassed sequences of the MHC-neo^r transgene internal to the oligonucleotide primers used for PCR amplification. Amplification of control EA3 DNA produced an appropriately sized band which hybridized to the transgene probe on the Southern blot (Fig. 5). An identical band was observed in amplification products of DNA prepared from DYS1-positive sections, but not in the amplification products of nonengrafted samples (Fig. 5). These results provided a second independent marker for the presence of engrafted G418-selected cardiomyocytes. The results of the graft analyses are summarized in Table III.

Discussion

This report describes a simple genetic modification which permitted the generation of essentially pure cultures of cardiomyocytes from differentiating ES cells. Cardiomyocytes expressing an MHC-neo^r transgene survived G418 selection and remained stable in culture for extended periods of time. In contrast, noncardiomyocyte cell lineages derived from the parental ES cell did not express the fusion gene and consequently were eliminated by G418 selection. This procedure reproducibly yielded cardiomyocyte cultures which were > 99% pure, as based on sarcomeric myosin immunoreactivity in isolated cell preparations. Expression of the MHC-neo^r transgene did not impact negatively on cardiogenic differentiation, as evidenced by the spontaneous contractile activity of selected cells. This observation was supported by both immunofluorescence and ultrastructural analyses, which documented the presence of well-developed myofibers in the selected cells. Intercalated discs were observed to couple juxtaposed selected cardiomyocytes, consistent with the observation that adjacent cells exhibited synchronous contractile activity. Collectively these data indicate that the selection process gives rise to highly differentiated cells. Furthermore, the selected cardiomyocytes were able to form stable intracardiac grafts, with the engrafted cells aligned with host cardiomyocytes.

The differentiated status of ES-derived cardiomyocytes is well established. Molecular, cytological, electrophysiological, and contractile analyses have demonstrated that ES-derived cardiomyocytes exhibit many of the physical and molecular attributes observed in typical cardiomyocytes (9–18). Although the analyses of selected EA3-derived cardiomyocytes presented here were of a more limited scope, all of the markers examined were consistent with a highly differentiated cardiomyocyte preparation. A priori, one would anticipate that the entire spectrum of differentiated characteristics seen in naive ES-derived cardiomyocyte preparations would also be present in the EA3-selected cells. Refinement of the selection procedure (i.e., selection of atrial versus ventricular cardiomyocytes, or selection of conduction system versus working cardiomyocytes) should be possible simply by choosing an appropriately cell type–restricted promoter to target aminoglycoside phosphotransferase expression. Combinatorial approaches using multiple promoters and selectable markers also can be envisioned. The observation that cells with attributes similar to atrial or ventricular cardiomyocytes were present in G418-selected cultures bodes well for the feasibility of isolating specific cardiomyocyte subtypes.

The approach presented here can generate as many as 10^6 ES-derived cardiomyocytes with an initial input of 10^6 stem cells. However, the inherent variability of cardiogenic induction in differentiating ES cultures can have a significant impact on the ultimate yield in a given preparation. Preliminary studies have indicated that many factors can influence the cardiomyocyte yield. These factors include serum content, length of growth in suspension culture, and initial embryoid body plating density. Thus, while the yield of G418-selected cardiomyocytes currently is somewhat variable, it is likely that optimization and standardization of the induction, culture, and selection protocols will result in improved and more reproducible preparations.

Cellular engraftment is emerging as a viable approach to augment myocyte number in adult hearts. To date several myocyte preparations have been grafted successfully into the adult myocardium. These include AT-1 tumor cardiomyocytes (1, 2), both naive and genetically engineered myoblasts (3–6), and fetal cardiomyocytes (7, 8). The limited studies reported to date suggest that fetal cardiomyocyte engraftment may hold the greatest potential to effect myocardial repair, as the engrafted donor cells were observed to form nascent intercalated discs with host cardiomyocytes (7, 8). While it remains to be established if any form of cellular engraftment can effect myocardial repair (see reference 26 for a critical review), it is virtually certain that human fetal donor cardiomyocytes cannot be obtained in sufficient numbers for use in a clinical setting. As such, identification of an alternative source of donor cardiomyocytes would constitute an important advancement. The observation that selected EA3 cardiomyocytes form stable intracardiac grafts raises the possibility that ES-derived cells may constitute a viable alternative source of donor cells. Im-

portantly, the frequency of successful engraftment with EA3 cardiomyocytes was similar to that observed with fetal murine cardiomyocytes (7, 8). Engrafted myofiber-containing donor cells were observed to be aligned and tightly juxtaposed with host cardiomyocytes. The generation of stable intracardiac grafts in the absence of teratoma formation further attests to both the cardiomyocyte purity and cell cycle withdrawal attained with the selected cultures. Collectively these observations suggest that ES-derived cardiomyocyte preparations may be a useful surrogate for fetal cardiomyocytes in cardiac engraftment procedures.

The EA3 cells also may prove to be a useful cell culture resource. Given the limited proliferative capacity of naive mammalian cardiomyocytes (27), most in vitro experiments have used primary cultures of fetal or neonatal cells. Although the utility of such cell preparations is well established, the cultures are cumbersome to generate and frequently can be overrun by fibroblasts. Efforts to generate myocardial cell lines have focused largely on the targeted expression of oncogenes, either in cultured cardiomyocytes or in the myocardium of transgenic animals (for reviews see references 28 and 29). Although numerous cell culture resources have been generated in this manner, to date no established cell lines exhibiting contractile activity after repeated passage have been reported. While the EA3 ES cells can be cultured and passaged in the undifferentiated state, it should be stressed that the proliferative capacity of the nascent cardiomyocytes is limited. Indeed, previous analyses of nonselected cultures have indicated that ES-derived cardiomyocytes withdraw from the cell cycle in a temporal pattern roughly similar to that observed for cardiomyocytes during normal murine development (18). Moreover, ES-derived cardiomyocyte cell cycle withdrawal was accompanied by a shift toward multinucleation, a hallmark of cardiomyocyte cell cycle withdrawal in vivo. While the selection procedure described here does not provide a source of continuously proliferating cardiomyocytes, the ability to propagate the parental EA3 cell provides a continuously renewable ex vivo source of pure cardiomyocyte cultures. Importantly, selected cardiomyocytes with spontaneous contractile activity can be cultured for extended periods of time without encroachment by other cell lineages.

The selection procedure described here should be applicable to all ES-derived cell lineages, provided that suitable cell type–specific promoters are available. Given that numerous cell lineages of ecto-, endo-, and mesodermal origin are represented in differentiating ES cultures (9), it is likely that ES-derived cellular transplantation strategies can be extended to other organ systems. To date, ES lines capable of cardiogenic differentiation have been generated in a number of species, including mouse, rat, rabbit, mink, pig, and most recently primates (30). If cardiomyocyte engraftment proves to be of therapeutic value, the generation of cardiogenic human ES cell lines would preclude the requirement for either human fetal or xenotrophic tissue. The existence of pluripotent human embryonic carcinoma cell lines is encouraging with regards to the prospects of generating cardiogenic human ES cells.

Acknowledgments

We thank H. Wang and D. Field for excellent technical assistance, Rick Mortensen (Harvard Medical School, Cambridge, MA) for the PGK-hygromycin transgene, J. Robbins (University of Cincinnati School of Medicine, Cincinnati, OH) for the mouse α-cardiac MHC promoter, Austin G. Smith (University of Edinburgh, Edinburgh, Scotland) for the LIF expression vector, Chris Glembotski (San Diego State University, San Diego, CA) for MA-APIII antibody, Jeff Robbins (CHRF, Cincinnati, OH) for the MLC-2a and MLC-2v oligonucleotides, and Tim Byers (Indiana University) for 6-10 antibody. MF20, D3, and 9D10 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under contract N01-HD-2-3144 from the National Institute of Child Health and Human Development.

This work was supported by grants HL-53505 and HL-52323 from the National Heart, Lung and Blood Institute. This work was done during the tenure of an Established Investigatorship from the American Heart Association (L.J. Field). M.G. Klug was supported by a predoctoral fellowship from the American Heart Association, Indiana Affiliate.

References

1. Koh, G.Y., M.H. Soonpaa, M.G. Klug, and L.J. Field. 1993. Long-term survival of AT-1 cardiomyocyte grafts in syngeneic myocardium. *Am. J. Physiol.* 264:H1727–H1733.

2. Delcarpio, J.B., and W.C. Claycomb. 1995. Cardiomyocyte transfer into the mammalian heart: cell-to-cell interactions in vitro and in vivo. *Ann. NY Acad. Sci.* 752:267–285.

3. Koh, G.Y., M.G. Klug, M.H. Soonpaa, and L.J. Field. 1993. Differentiation and long-term survival of C2C12 myoblast grafts in heart. *J. Clin. Invest.* 92:1548–1554.

4. Chiu, R.C., A. Zibaitis, and R.L. Kao. 1995. Cellular myoplasty: myocardial regeneration with satellite cell implantation. *Ann. Thorac. Surg.* 60:12–18.

5. Robinson, S.W., P.C. Cho, M.A. Acker, and P.D. Kessler. 1994. Arterial delivery of skeletal myoblasts to the heart. *J. Cell. Biochem.* 18D:531*a* (Abstr.)

6. Koh, G.Y., S.J. Kim, M.G. Klug, K. Park, M.H. Soonpaa, and L.J. Field. 1995. Targeted expression of TGF-β1 and neovascularization in hearts bearing intracardiac grafts. *J. Clin. Invest.* 95:114–121.

7. Soonpaa, M.H., G.Y. Koh, M.G. Klug, and L.J. Field. 1994. Formation of nascent intercalated discs between grafted fetal cardiomyocytes and host myocardium. *Science (Wash. DC).* 264:98–101.

8. Koh, G.Y., M.H. Soonpaa, M.G. Klug, H.P. Pride, D.P. Zipes, B.J. Cooper, and L.J. Field, 1995. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. *J. Clin. Invest.* 96:2034–2042.

9. Doetschman, T.C., H. Eistetter, M. Katz, W. Schmidt, and R. Kemler. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27–45.

10. Sanchez, A., W.K. Jones, J. Gulick, T. Doetschman, and J. Robbins. 1991. Myosin heavy chain gene expression in mouse embryoid bodies. An in vitro developmental study. *J. Biol. Chem.* 266:22419–22426.

11. Muthuchamy, M., L. Pajak, L. Howles, T. Doetschman, and D.F. Wieczorek. 1993. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Mol. Cell. Biol.* 13:3311–3323.

12. Miller-Hance, W.C., M. LaCorbiere, S.J. Fuller, S.M. Evans, G. Lyons, C. Schmidt, J. Robbins, and K.R. Chien. 1993. In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *J. Biol. Chem.* 268: 25244–25252.

13. Ganim, J.R., W. Luo, S. Ponniah, I. Grupp, H.W. Kim, D.G. Ferguson, V. Kadambi, J.C. Neumann, T. Doetschman, and E.G. Kranias. 1992. Mouse phospholamban gene expression during development in vivo and in vitro. *Circ. Res.* 71:1021–1030.

14. Boer, P.H. 1994. Activation of the gene for type-b natriuretic factor in mouse stem cell cultures induced for cardiac myogenesis. *Biochem. Biophys. Res. Commun.* 199:954–961.

15. Metzger, J.M., W.-I. Lin, and L.C. Samuelson. 1994. Transition in cardiac contractile sensitivity to calcium during the in vitro differentiation of mouse embryonic stem cells. *J. Cell Biol.* 126:701–711.

16. Maltsev, V.A., J. Rohwedel, J. Hescheler, and A.M. Wobus. 1993. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech. Dev.* 44:41–50.

17. Wobus, A.M., G. Wallukat, and J. Hescheler, 1991. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca++ channel blockers. *Differentiation.* 48:173–182.

18. Klug, M.G., M.H. Soonpaa, and L.J. Field. 1995. DNA synthesis and multinucleation in embryonic stem cell-derived cardiomyocytes. *Am. J. Physiol.* 269:H1913–H1921.

19. Gulick, J., A. Subramaniam, J. Neumann, and J. Robbins. 1991. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J. Biol. Chem.* 266:9180–9185.

20. Mortensen, R.M., D.A. Conner, S. Chao, L.A. Geisterfer, and J.G. Seidman. 1992. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* 12:2391–2395.

21. Smith, A.G. 1991. Culture and differentiation of embryonic stem cells. *J. Tissue Culture Methods.* 13:89–94.

22. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Press, Cold Spring Harbor, NY.

23. Steinhelper, M.E., K. Cochrane, and L.J. Field. 1990. Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension (Dallas).* 16:301–307.

24. Zeller, R., D. Bloch, B.S. Williams, R.J. Arceci, and C.E. Seidman. 1987. Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes & Dev.* 1:693–698.

25. Danko, I., V. Chapman, and J.A. Wolff. 1992. The frequency of revertants in mdx mouse genetic models for Duchenne muscular dystrophy. *Pediatr. Res.* 32:128–131.

26. Soonpaa, M.H., M.G. Klug, H. Nakajima, H. Nakajima, and L.J. Field. 1996. Potential approaches for cell-mediated myocardial repair. *In* Unstable Coronary Artery Disease: Pathophysiology, Diagnosis and Treatment. R.W. Wilensky, editor. Kluwer Academic Press, New York. In press.

27. Rumyantsev, P.P. 1991. Reproduction of cardiac myocytes developing in vivo and its relation to processes of differentiation. *In* Growth and Hyperplasia of Cardiac Muscle Cells. B.M. Carlson, editor. Harwood Academic Press, New York. 70–157.

28. Field, L.J. 1993. Transgenic mice in cardiovascular research. *Ann. Rev. Physiol.* 55:97–114.

29. Daud, A.I., and L.J. Field. 1993. Interaction between host proteins and SV40 large T-antigen in transformed cardiomyocytes. *Heart Failure.* 9:183–198.

30. Thomson, J.A., J. Kalishman, T.G. Golus, M. Durning, C.P. Harris, R.A. Becker, and J.P. Hearn. 1995. Isolation of a primate embryonic stem cell. *Proc. Natl. Acad. Sci. USA.* 92:7844–7848.

STIC-ILL

AU 5094905

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

372881

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

8907264

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic

STIC-ILL

09/686,903

From: Chen, Shin-Lin
Sent: Wednesday, November 21, 2001 5:53 PM
To: STIC-ILL
Subject: articles

372879

Please provide the following articles ASAP. Thanks!
Serial No. 09/686,880.

L14 ANSWER 6 OF 8 MEDLINE
AU Bertolini F; Corsini C; Lazzari L; Soligo D; De Monte L; Ward M; Bank A; Sirchia G
TI Gene transfer-mediated generation of drug-resistant hemopoiesis.
SO LEUKEMIA AND LYMPHOMA, (1996 Mar) 21 (1-2) 17-23. Ref: 31

L14 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2001 ISI (R)
AU BERTOLINI F (Reprint); CORSINI C; LAZZARI L; SOLIGO D; DEMONTE L; WARD M; BANK A; SIRCHIA G
TI GENE TRANSFER-MEDIATED GENERATION OF DRUG-RESISTANT HEMATOPOIESIS
SO LEUKEMIA & LYMPHOMA, (MAR 1996) Vol. 21, No. 1-2, pp. 17.

L14 ANSWER 8 OF 8 MEDLINE DUPLICATE 2
AU Islam A; Glomski C; Henderson E S
TI Evidence for proliferation and differentiation of endosteal cells into hemopoietic cell lines in short-term liquid culture.
SO JOURNAL OF MEDICINE, (1989) 20 (2) 113-22.

L15 ANSWER 3 OF 11 MEDLINE
AU Verma S; Woffendin C; Bahner I; Ranga U; Xu L; Yang Z Y; King S R; Kohn D B; Nabel G J
TI Gene transfer into human umbilical cord blood-derived CD34+ cells by particle-mediated gene transfer.
SO GENE THERAPY, (1998 May) 5 (5) 692-9.
Journal code: CCE; 9421525. ISSN: 0969-7128.

L15 ANSWER 5 OF 11 MEDLINE
AU McWhir J; Schnieke A E; Ansell R; Wallace H; Colman A; Scott A R; Kind A J
TI Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.
SO NATURE GENETICS, (1996 Oct) 14 (2) 223-6.
Journal code: BRO; 9216904. ISSN: 1061-4036.

2769081

L15 ANSWER 6 OF 11 MEDLINE
AU Klug M G; Soonpaa M H; Koh G Y; Field L J
TI Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts.
SO JOURNAL OF CLINICAL INVESTIGATION, (1996 Jul 1) 98 (1) 216-24.
Journal code: HS7; 7802877. ISSN: 0021-9738.

L15 ANSWER 7 OF 11 MEDLINE
AU Pantazis P; Dejesus A; Early J; Rodriguez R; Chatterjee D; Han Z; Wyche J; Giovanella B
TI Development of human leukemia U-937 cell sublines resistant to doxorubicin: induction of differentiation and altered sensitivities to topoisomerase-directed drugs.
SO ANTICANCER RESEARCH, (1995 Sep-Oct) 15 (5B) 1873-81.
Journal code: 59L; 8102988. ISSN: 0250-7005.

L15 ANSWER 8 OF 11 MEDLINE DUPLICATE 1
AU Nakano T; Kodama H; Honjo T
TI Generation of lymphohematopoietic cells from embryonic